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Award Number: W81XWH-08-1-0087

TITLE: Preclinical Studies of Signaling Pathways in a Mutant Mouse Model of Hormone-Refractory Prostate Cancer

PRINCIPAL INVESTIGATOR: Dr. Corry Abate-Shen

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

REPORT DATE: February 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-02-2011	Final	28 JAN 2008 - 27 JAN 2011

4. TITLE AND SUBTITLE

Preclinical Studies of Signaling Pathways in a Mutant Mouse Model of Hormone-Refractory Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0087

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Corry Abate-Shen

E-Mail: abate@cabm.rutcabateshen@columbia.edugers.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, NY 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have been investigating targeted therapies for the treatment of advanced prostate cancer using a genetically-engineered mouse model of the disease. Based on previous studies, we performed pre-clinical studies to examine the consequences of combinatorial inhibition of these signaling pathways for prostate tumorigenesis an androgen-independence. We found that combination therapy using Rapamycin, an inhibitor of mTOR, and PD0325901, a MEK inhibitor, is potently anti-tumorigenic in Nkx3.1; Pten mutant mice, particularly in contexts of limiting androgens. Furthermore, we find that these signaling pathways are coordinately de-regulated during prostate cancer progression in humans, as evident by our comprehensive analyses of their status in human tissue microarrays. Based on these pre-clinical studies in the mutant mice, and our supporting data from human prostate cancer, we propose that combination therapy targeting the Akt/mTOR kinase and Erk Map kinase signaling pathways may be effective for treatment of a broad spectrum of patients with advanced prostate cancer, particularly when used in conjunction with androgen deprivation therapy.

15. SUBJECT TERMS
Rapamycin, mTOR, PD0325901, MEK inhibitor, Nkx3.1, Pten, Akt/mTOR, Erk Map

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	59	**19b. TELEPHONE NUMBER** *(include area code)*

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1. Introduction

The prostate is critically dependent on androgen receptor signaling for all stages of its normal growth and development as well as all stages of cancer. Accordingly, androgen-deprivation has been widely used for the treatment of prostate cancer. Although this therapy initially results in tumor regression and decreased PSA levels, most patients develop hormone-refractory tumors that are resistant to available treatments. Counter-intuitively, most hormone-refractory tumors remain dependent on androgen receptor (AR) signaling and have devised mechanisms to bypass the need for testicular androgens for tumor growth. Thus, it is essential to understand how these tumors arise in the absence of testicular androgens and to use this understanding to develop strategies to control the development of hormone-refractory disease. Our proposal is based on published studies from us and others showing that two key signaling pathways, namely the Akt/mTOR and B-Raf/Erk MAP kinase pathways, promote hormone-refractory prostate cancer in an AR-dependent manner. We **hypothesize** that combination therapy for the PI3Kinase/Akt/mTOR and B-Raf/MEK/Erk MAP kinase pathways will be effective for treating or preventing hormone-refractory prostate cancer.

We had proposed to: (1) **Targeting the Akt/mTOR and B-Raf/Erk MAP kinase pathways —** investigate the consequences of combinatorial inhibition of these signaling pathways at distinct steps in cell culture and in a novel human organ culture assay using available pharmacological agents. (2) **Inhibition of Akt/mTOR and B-Raf/Erk MAP kinase pathways in pre-clinical studies —** evaluate the consequences of inhibiting Akt/mTOR and B-Raf/MAP signaling following androgen-deprivation in pre-clinical studies in a mouse model of hormone-refractory prostate cancer. These pre-clinical studies will test the **hypothesis** that pharmacological manipulation of the Akt/mTOR and B-Raf/MAP kinase pathways will control or prevent hormone-refractory disease.

2. Body

2.a. Summary of work accomplished over the entire funding period

Because of its central relevance for prognosis of patients with prostate cancer, we have focused on investigating the molecular mechanisms underlying castration-resistant disease and the identification of new molecular targets for therapeutic intervention. In comparative analyses of prostate cancer progression in intact versus castrated *Nkx3.1; Pten* mutant mice, we found that the Akt/mTOR and Erk MAP kinase signaling pathways are coordinately up-regulated during cancer progression, particularly in androgen-independent tumors [1]. Furthermore, we showed that these two signaling pathways cooperate to promote androgen-independent tumor growth *in vivo*, consistent with studies in human prostate cancer cells [2].

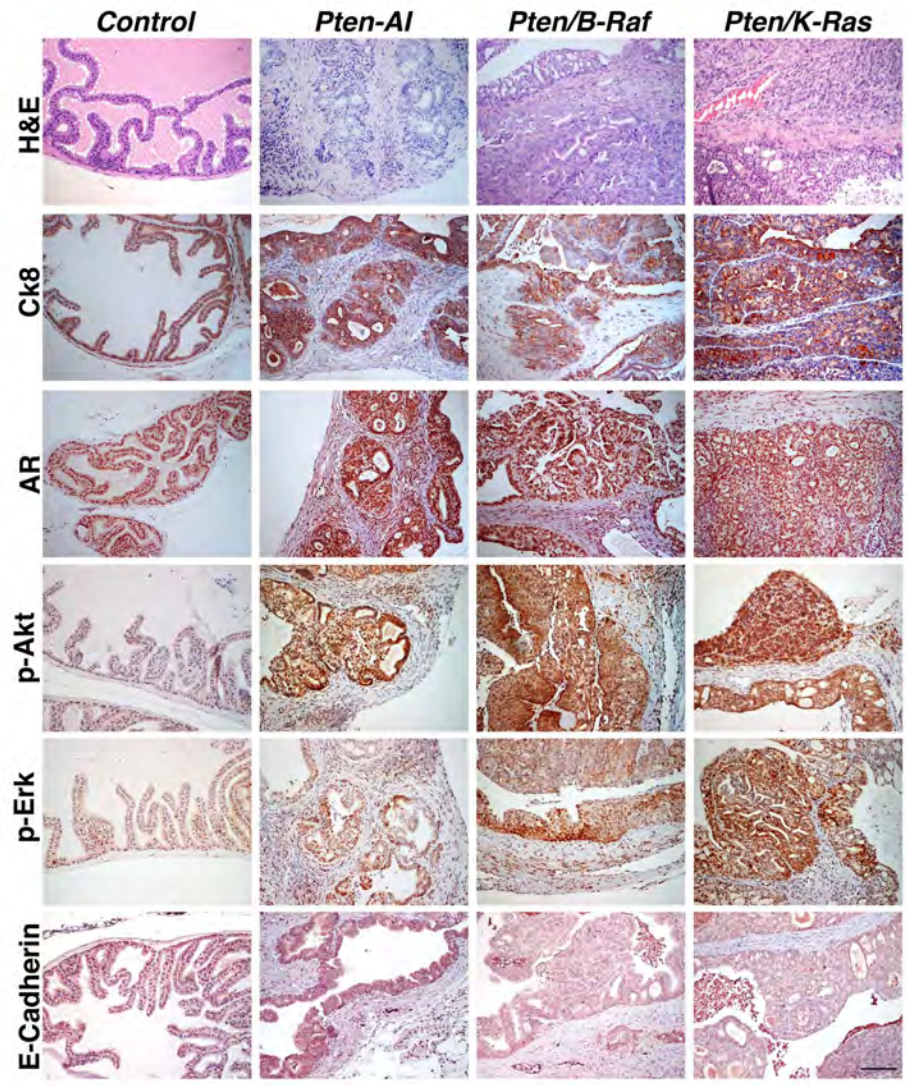
Based on these observations, we performed **pre-clinical studies** to test whether combinatorial inhibition of these pathways could block cancer progression, in androgen-independent contexts [3]. For these studies, we used rapamycin to target the Akt/mTOR signaling pathway and a Pfizer MEK inhibitor (PD035901) to target MEK/MAP kinase signaling, and we examined pS6 and pErk respectively as downstream read-outs of pathway activity. Using our CASP cells, we found that these agents act cooperatively to promote cell toxicity, mediated in part by the pro-apoptotic regulator, Bim.

We next performed preclinical studies to evaluate the efficacy of this combination *in vivo*. To do so, we treated the *Nkx3.1; Pten* mutant mouse model with combination therapy using rapamycin and PD035901 profoundly affected the growth of androgen-independent prostate tumors. In particular, delivery of these agents in combination (but not individually) for a period of one month resulted in a significant reduction in the occurrence of PIN/cancer lesions, as well as a 2.5 fold reduction in tumor size and 14 fold decrease in proliferation. Furthermore, analyses of human tissue microrarrays revealed that 25% of human prostate cancer cases display activation of both Akt/mTOR and MEK/MAPK pathways [3]. These findings suggest that a sizable population of prostate cancer patients may benefit from combination treatment targeting these two signaling pathways. In ongoing studies, we are pursuing pre-clinical studies in this mouse model to evaluate the best combination(s) of pathway-blocking agents, which should ultimately guide the development of new clinical trials for patients with castration-resistant prostate cancer.

2.b. Summary of work accomplished in the last year of funding

To address more clinically relevant questions, we have developed “next-generation” models are based on a unique *Nkx3.1*^{CreERT2} allele, which expresses a fusion protein of Cre with a mutated estrogen receptor (ER^{T2}) under the control of the *Nkx3.1* promoter; the resulting $CreER^{T2}$ fusion protein is completely inactive *in vivo*, but rapidly activated by administration of tamoxifen resulting in Cre-mediated recombination in up to 40% of prostate epithelial cells, with **no detectable background recombination in non-prostate tissues** and **no**

adverse consequences from the limited exposure to tamoxifen [4]. Notably, this CreER^{T2} allele achieves recombination in a luminal cell of origin for prostate cancer [4], which is highly relevant as most human prostate cancers have a luminal phenotype.



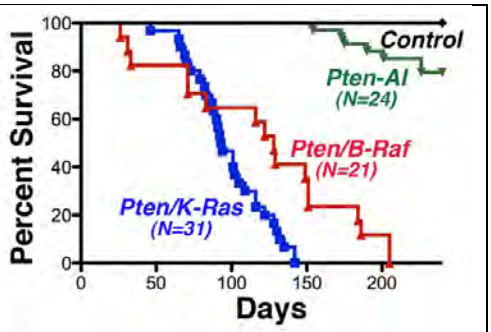


Figure 1. Prostate cancer phenotypes of "next generation" mouse models. ***Left*** Phenotype analyses: Mice were injected with vehicle (not induced) or tamoxifen at 2 months and analyzed at 9 months (Control and *Pten-AI*) or 4 months (*Pten/K-Ras* and *Pten/B-Raf*). Histological sections of the anterior prostate show H&E or immunohistochemical staining with the indicated antibodies. Note the poorly differentiated cancer phenotype in each of the tumor-prone (tamoxifen-induced) mice. These tumors express CK8, a luminal cell marker, in the prostatic epithelium as well as androgen receptor (AR) and activated forms of Akt (p-Akt) and Erk (p-Erk). E-Cadherin is is localized to the membrane in *Pten-AI* and control mice, but has reduced expression and diffuse localization in the cytoplasm in the *Pten/K-Ras* and *Pten/B-Raf* tumors. Data shown are representative sections from analyses N=10 for H&E and N=5 for IHC. Procedures are as in [1]. ***Top*** Survival curve: Mice were monitored for survival for the indicated period, showing the number of mice monitored in each group. The significance of survival of *Pten/K-Ras* and *Pten/B-Raf* versus *Pten-AI* mice is *P<0.0001*. Number of mice indicated.

Prostate cancer phenotype*:** Using this *Nkx3.1*^{CreERT2} driver, we have generated a new series of GEM models having: *(i)* a conditional *Pten* allele [5]; *(ii)* a Cre-activatable mutant *K-ras* allele (*K-ras*^{G12D}) [6]; and *(iii)* a Cre-activatable mutant *B-Raf* allele (*B-Raf*^{V600E}) [7] (see Table 1). Following tamoxifen induction, the androgen-intact as well as androgen-deprived (castrated) mice develop prostate tumors with varying phenotypic severity, lethality, and occurrence of metastases, *which are completely dependent upon tamoxifen induction* (Figs. 1 to 3, Table 1). In particular, mice lacking *Pten* function in the prostate (*Nkx3.1*$^{CreERT2/+}$; *Pten*$^{flox/flox}$) develop high-grade PIN with areas of invasion by 6 months of age, and poorly differentiated adenocarcinoma in mice older than 12 months of age, while androgen deprivation (*i.e.*, surgical removal of the testes [8]) results in the emergence of castration-resistant prostate tumors (Fig. 1). (Hereafter the castration-resistant *Nkx3.1*$^{CreERT2/+}$; *Pten*$^{flox/flox}$ mice are termed ***Pten-AI). The *Pten-AI* mice develop large tumors (~2-7 mm) that can readily be visualized by MRI imaging and display histological features of highly aggressive and poorly differentiated histology (Fig. 1, data not shown). Nonetheless, there is no adverse effect on their survival, as most of the *Pten-AI* mice live for up to two years (Fig. 1), consistent with other GEM models based on prostatic-specific deletion of *Pten (e.g.,* [9, 10]).

In contrast, mice lacking *Pten* together with activation of either the *B-Raf* or *K-Ras* alleles (*Nkx3.1*$^{CreERT2/+}$; *Pten*$^{flox/flox}$; *B-Raf*$^{LSL/+}$ or *Nkx3.1*$^{CreERT2/+}$; *Pten*$^{flox/flox}$; *K-Ras*$^{LSL/+}$; hereafter termed ***Pten/B-Raf*** and ***Pten/K-Ras***, respectively*)* display highly aggressive prostate tumors that result in ~100% lethality by 6 or 4 months of age, respectively (Fig. 1). Compared to the *Pten-AI* mice, the prostate tumors in *Pten/B-Raf* and *Pten/K-Ras* mice have more poorly differentiated histology. Notably, the prostate tumors in each of these mice are primarily luminal, as evident by robust staining for the luminal cytokeratin CK8, in contrast to the restricted

staining for basal cytokeratins (*e.g.,* CK5) and rare staining for neuroendocrine markers (*e.g.*, synaptophysin) (Fig. 1). These tumors are highly proliferative and express androgen receptor (AR) as well as phosphorylated (activated) forms of Akt and MAP kinase, the latter being significantly elevated in the *Pten/B-Raf* and *Pten/K-Ras* prostate, consistent with *Raf* or *Raf* activation of this pathway (Fig. 1). Interestingly, while the *Pten-AI* mice have robust membrane-localized staining of E-Cadherin, the *Pten/B-Raf* and *Pten/K-Ras* have reduced staining that is diffusely localized (Fig. 1), potentially indicative of EMT**.**

Table 1: Summary of the metastatic phenotype in "next generation" mouse models

Relevant models[a]		Description of prostate and metastatic phenotype	Metastases[b]		
Strain name	**Abbrev.**		**DTC**	**Lymph**	**Distant**
Nkx3.1$^{CreERT2/+}$; *Pten*$^{flox/flox}$*(uninduced)*	Control	Normal prostate; no metastases	0%, 0/5	0%, 0/5	0%, 0/5
Nkx3.1$^{CreERT2/}$; *Pten*$^{flox/flox}$ *(castrated)*	Pten-AI	Non-lethal castration-resistant prostate cancer; Lymph node metastases only	0% 0/16	60%, 3/5	0%, 0/10
Nkx3.1$^{CreERT2/}$; *Pten*$^{flox/flox}$; *B-Raf*$^{LSL/+}$	Pten/B-Raf	Lethal prostate cancer; Metastases to lymph node; infrequent hematogenous metastases to lung, liver	20%, 2/10	100%, 5/5	20%, 2/10
Nkx3.1$^{CreERT2/}$; *Pten*$^{flox/flox}$; *Kras*$^{LSL/+}$	Pten/K-Ras	Lethal prostate cancer; Metastases to lymph node; infrequent hematogenous metastases to lung, liver	80%, 15/19	100%, 5/5	71%, 12/17

Legend: a) We have analyzed all combinations of these mouse alleles, including the androgen-intact Pten mice and the K-Ras and B-Raf single mutants; in interests of brevity only those used in this proposal are described. Additional controls include *Nkx3.1*$^{+/+}$; Pten$^{flox/flox}$ (tamoxifen induced). ****In all cases,* androgen deprivation accelerates both the cancer and metastatic phenotype.** b) Metastases to lungs and liver were scored by histological inspection as well as two IHC markers, CK8 and AR. DTC refers to disseminated tumor cells. The % and number of cases for each is indicated.

***Metastases phenotypes*:** While each of the *Pten-AI*, *Pten/B-Raf* and *Pten/K-Ras* models, display frequent metastases to lymph nodes, particularly the lumbar node, which is nearest to the prostate, they display differing degrees of hematogenous metastases to distant organs, including lung and liver. In particular, *Pten-AI* mice do not (or rarely) develop distant metastases, consistent with previous studies (*e.g.,* [9]) while *Pten/B-Raf* mice display distant metastases in ~ 20% of cases, and *Pten/K-Ras* mice in > 70% of the cases (Table 1, Fig. 2).

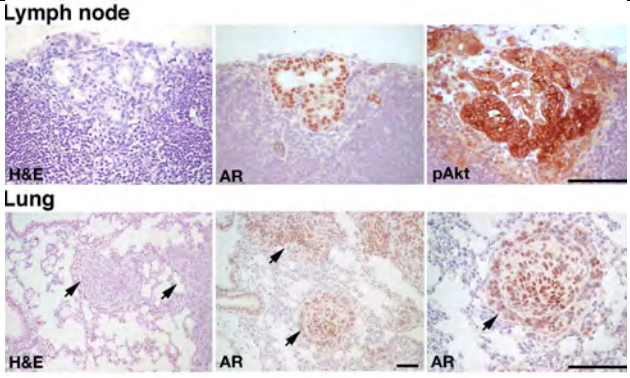


Figure 2. M**etastastic phenotype:** H&E and IHC images from lumbar lymph node and lungs of *Pten/K-Ras* mice.

Furthermore, the occurrence of distant metastases was well-correlated with the number of cases having **disseminated tumor cells** in the bone marrow. We used two assays to evaluate the occurrence of disseminated tumor cells in bone marrow:
(1) a PCR-based approach to quantify the targeted allele; and *(2)* direct visualization of disseminated cells by immunofluorescence imaging of lineage-marked cells (YFP) plus tumor markers, such as activated Akt (Fig. 3). In particular, disseminated cells were detected in **none** of the *Pten-AI* mice (0/16), **few** in the *Pten/B-Raf* mice (2/10), and **many** of the *Pten/K-Ras* mice (15/19) (Fig. 1). ***In summary****, the Pten-AI, Pten/B-Raf, and Pten-K-Ras mice provide a progressive series of prostate cancer phenotypes with an increasing tendency to display disseminated tumor cells, a similar increasing tendency of distant metastases, and an increasing tendency of lethality that is well-correlated with the occurrence of metastases.* As such, they are ideally suited for our proposed analyses of the molecular and cellular events involved in the evolution of metastatic prostate cancer.

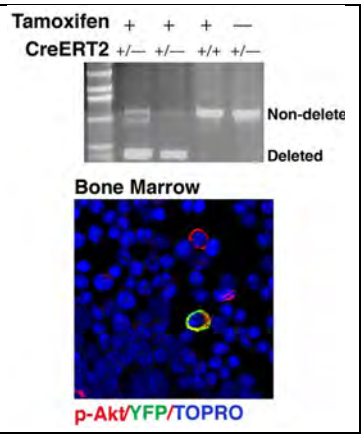


Figure 3. Disseminated tumor cells in bone marrow. *Top*. PCR-based assay for detecting recombined alleles in bone marrow. Note **absence** of the deleted allele in controls not induced with tamoxifen or lacking the CreERT2 allele, confirming that the mutant allele is from prostate tumor cells and not due to ectopic deletion of Pten in bone marrow. *Bottom*: Image of disseminated cell in bone marrow of *Pten/K-Ras* mice, expressing p-Akt and lineage-marked YFP (arrow).

2.c. Preclinical analyses

Using our "first-generation" *Nkx3.1; Pten* mutant mice, we established that the Akt/mTOR and Erk MAP kinase signaling pathways are coordinately up-regulated in castration-resistant prostate tumors and cooperate

to promote castration-resistant tumor growth *in vivo* [1], while combinatorial inhibition of these signaling pathways blocks castration-resistant prostate tumor growth [3]. Analyses of our "next generation" GEM models has now allowed us to pursue preclinical studies that address more clinically-relevant issues, such as whether combinatorial targeting of these pathways improves survival and/or alleviates metastasis. In particular, preclinical studies using our new GEM models have shown that combination therapy using Rapamycin to target the Akt/mTOR pathway and a Pfizer MEK inhibitor (PD035901) to target the MEK/MAP kinase pathway [3] (hereafter termed **RAP + PD**) reduces tumor burden in the *Pten-AI, Pten/B-Raf*, and *Pten/K-Ras* mouse models as evident by analyses of tumor weights (9.15 fold, *P*=0.0067, N=12/group), histological inspection of the prostate phenotype, and MRI tumor volumetric analyses (Fig. 4). Notably, RAP + PD was more effective at reducing tumor burden than standard-of-care chemotherapy, such as docetaxel [11]. Alternative inhibitors of these signaling pathways now in clinical trials, such as a combination from Merck that target Akt and mTOR (MK2206 and MK8669) [12] were also effective for reducing tumor burden (Fig. 4).

In striking contrast to the effect on *tumor burden*, the consequences of RAP + PD on *metastatic burden* were profoundly different *immediately* following the treatment versus *delayed* post-treatment (Fig. 4). Specifically, *immediately* following treatment, the incidence of lung metastases was undetectable in the drug-treated mice compared to the vehicle group (N=0/20), whereas the *delayed* post-treatment mice had nearly as many lung metastases as the vehicle mice (N=15/20). Interestingly, the incidence of disseminated tumor cells was reduced but not abrogated *immediately* following treatment but was nearly the same as vehicle in the *delayed* post-treatment group. Thus, agents such as RAP + PD may have a differential response on the primary tumor versus metastases.

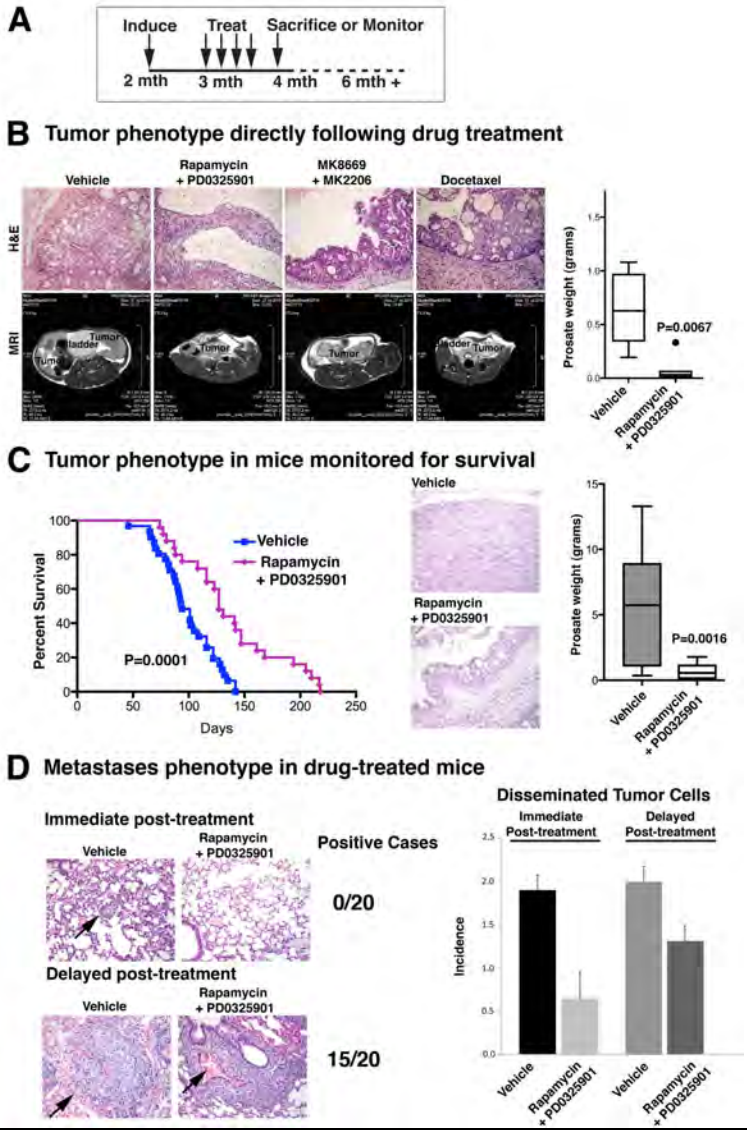


Figure 4. Preclinical analyses of GEM mice.
(A) *Strategy* Tumors were induced in *Pten/K-Ras* mice by transient delivery of tamoxifen (at 2 months). Mice were then treated with vehicle or the indicated agents starting at 3 moths for 1 month followed by sacrifice (at 4 months) or monitoring for survival (up to ~7 months). *The survival cohort is referred to as the delayed post-treatment group*. NOTE: All GEM models (including the *Pten-AI, Pten/B-Raf* and relevant controls) were analyzed; however, in the interests of brevity, only data for the *Pten/K-Ras* mice are shown as this model is most relevant for our proposed studies.
(B) Prostate phenotype immediately following treatment. Representative sections showing histology (H&E), MRI images, and prostate weights.
(C) Prostate phenotype in delayed post-treatment (the survival cohort). Mice treated with RAP + PD show improved survival relative to mice treated with Vehicle. Prostate histology and weights of the delayed post-treatment group show persistent tumor remission.
(D) Metastatic phenotype: immediately following RAP + PD treatment, we observed no (0/20) cases of lung metastases compared to the Vehicle-treated mice; while the incidence in the delayed post-treatment group was nearly the same as the vehicle group (15/20). The incidence of disseminated tumor cells was reduced immediately following treatment but less so in the delayed post-treatment group.
Methods: Dosage and treatment schedule is as follows: Rapamycin (10 mg/kg) delivered IP in combination with PD0325901 (10 mg/kg) delivered PO 3 days a week. MK-8669 (10 mg/kg) delivered by IP injection in combination with MK-2206 (120 mg/kg) delivered PO 3 days a week. Docetaxel (10 mg/kg) was delivered by IP injection twice a week. During the period of the experiment, mice were imaged to evaluate tumor volume using MRI imaging.

2.d. Statistical Analyses

In designing our experiments, we start with the premise that each trial will have one or more "treatment" groups and at least one "control" group; for simplicity, we consider only pair-wise comparisons between treatment and control. We also consider that there will be two main types of experimental designs: *(1)* where the outcome for each mouse is **binary**, such as "tumor" vs no "tumor; and *(2)* where the outcome is measured **longitudinally** over time, such as tumor volume. Different methods are needed for sample size calculations and analysis for these two types of experiments.

Binary outcome experiments*:* For simplicity, we will consider that the outcome is "tumor" or "no tumor", and we would like to compare these frequencies to determine whether there is a significant difference between the groups. Let p_c denote the frequency of control mice experiencing a tumor, and p_t denote the frequency of treatment mice experiencing a tumor, with the goal to compare p_c and p_t. Because sample sizes might be small, the Fisher's exact test is used to compare frequencies. For example, supposing that the tumor frequency is 50% among controls and 10% among treated mice, we would require 23 treated mice and 23 control mice to detect the difference $|p_c - p_t| = 0.40$.

Longitudinal experiments: In this case, some of the primary endpoints will be survival time and tumor volume. For each mouse, the area under the tumor volume curve (AUC) summarizes the tumor volume over time. Survival time is defined as the minimum of the time to death or maximum follow-up. In contrast to the clinical setting, the maximum follow-up time is constant across all animals, so special survival analysis techniques are unnecessary. The AUCs or survival times are ranked after pooling the data in the two groups, and a permutation test is employed to determine if the observed outcome between treatment groups occurred by chance [63]. In these experiments, randomization, either real or imagined, is an essential element for statistical comparisons. Under the null hypothesis that the difference between the two groups has no effect on the primary endpoint, there are 2n choose n distinguishable separations of the mice into two groups, each with equal probability under the null. The test statistic used to perform this comparison is the sum of the ranks in the experimental group after pooling the outcome data (*e.g.,* survival times or tumor volume AUCs). If the outcome data in the experimental population were greater, we would expect the ranks in the observed statistic to be large. To determine the size of the rank sum to achieve confidence that the endpoints in the two groups are different, a critical region is developed under the null permutation distribution, where each ordering has equal probability of occurrence. A power function is constructed under the semi-parametric specification — $1-F(x) = [1-G(x)]^{\gamma}$ — where F and G are the unknown distribution functions of the outcome data from the two groups, and g is defined as the odds an experimental group measurement is greater than a control group measurement. When the outcome measure is survival time, g represents the odds that an experimental group mouse will have a longer survival time than a control mouse. A similar parameter can be defined in terms of the AUC for tumor volume. Note that $g = 1$ corresponds to the null hypothesis of no difference in the survival times. For example, for 20 treated mice and 20 control mice, the odds would need to be 3:1 in favor of an experimental mouse having a longer survival time than a control mouse in order to have sufficient power.

Testing for synergy: Finally, we consider strategies to test whether a dose/schedule combination produces a synergistic effect. Each mouse under study will have its tumor growth measured over time, and the area under this tumor growth curve will be measured. Synergistic inhibition of tumor growth is defined as a dose/schedule combination producing on average a smaller AUC than predicted by the additive model that includes the dose and schedule separately. We describe this relationship through the inequality:

$$avg(V|D=d, S=s) < C + \{avg(V|D=d, S=0) - C\} + \{avg(V|D=0, S=s) - C\},$$

where V is the AUC, D and S represent the dose and schedule used in an experiment, and C is the average AUC in the control group $[C=avg(V|D=0, S=0)]$. To test for synergy, we will sample 2000 times from the distribution of AUCs within each of the four groups. From these samples we will compute the average AUC for each group, and determine the proportion of replicates where the inequality was not obtained using the equation above. This proportion is termed the achieved significance level (p-value).

2.e. Relevance of major findings to Statement of Work

Aim 1: Targeting the Akt/mTOR and B-Raf/MAP kinase pathways:

Task 1: Testing relevant drugs in cell culture for optimal dosage and for cytotoxicity (Months 1-9).

We obtained pharmacological inhibitors to the various components of the Akt and Erk MAP kinase signaling pathways and tested their optimal dosage in cell culture. We used mouse and human prostate cancer cell lines including androgen-dependent and androgen-independent cell lines (e.g., human — LNCaP, PC3, VCAP; mouse — CASP2.1, CASP1.1).

Status - completed:

Completed for Rapamycin and PD035901 and two agents obtained from Merck, namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

Task 2: Test the drugs individually and in combination for ability to inhibit androgen independence in cell culture (Months 4-12)

Status - completed:

Completed for Rapamycin and PD035901 as well as for a new combination agents obtained from Merck, namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

Task 3: Test the drugs individually and in combination using human organ culture (Months 12-24)

Status - completed:

We have had difficulty obtaining prostate tissues in sufficient amounts for organ culture study, so we shifted our focus to human cell lines and xenograft models.

Aim 2: Inhibition of Akt/mTOR and B-Raf/MAP kinase pathways in pre-clinical studies:

Task 1: Testing relevant drug combinations for optimal dosage and PD *in vivo* (Months 12-18).
Focusing on the most promising combinations of drugs identified in the studies in Aim 1, we investigates the optimal dosage individually and in combination in vivo.

Status - completed:

Completed for Rapamycin and PD035901 and for the new combination agents obtained from Merck, namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

Task 2: Test the drugs individually and in combination for ability to inhibit androgen independence *in vivo* (Months 18-36).

Status - completed:

Completed for Rapamycin and PD035901 in the original *Nkx3.1Pten* model as well as our new models of advanced prostate cancer (as described above). Additionally, we have completed these studies using the combination agents obtained from Merck, namely an Akt inhibitor (MK-2206) and a new mTOR inhibitor (MK-8669).

3. Key Research Accomplishments

- Combination therapy with Rapaymcin and PD035901 acts synergistically in prostate cancer cells by regulating Bim1
- Combination therapy with Rapaymcin and PD035901 in the *Nkx3.1; Pten* mutant mouse model represses castration-resistant prostate cancer
- Combination therapy with Rapaymcin and PD035901 in our new mutant mouse model of advanced prostate cancer represses prostate tumors and metastases
- Combination therapy with a new Merck combination in our new mutant mouse model of advanced prostate cancer represses prostate tumors

4. Reportable Outcomes

Kinkade, C.W., Castillo-Martin, M., Puzio-Kuter, A., Yan, J., Foster, T.H., Gao, H., Sun, Y., Ouyang, X., Gerald, W.L., Cordon-Cardo, C., and Abate-Shen, C. (2008) Targeting Akt/TOR and Erk MAPK signaling inhibits hormone-refractory prostate cancer in a pre-clinical mouse model. Journal of Clinical Investigations. 118: 3051-3064.

Shen, M.M. and **Abate-Shen, C** (2010) Molecular Genetics of Prostate Cancer: New prospects for old challenges. *Genes Dev.* 24:1967:2000.

5. Conclusion

Although most men diagnosed with early stage prostate cancer have favorable outcomes, those with advanced disease and particularly hormone-refractory prostate cancer eventually succumb to lethality since treatment options are limited. We have been investigating targeted therapies for advanced prostate cancer using genetically-engineered mouse models of the disease. Based on previous studies showing that the Akt/mTOR and Erk MAP kinase signaling pathways cooperate in prostate cancer progression, we performed pre-clinical studies to examine the consequences of combinatorial inhibition of these signaling pathways for prostate tumorigenesis in androgen-dependent and -independent contexts. We found that combination therapy using Rapamycin, an inhibitor of mTOR, and PD0325901, a MEK inhibitor, is potently anti-tumorigenic in castration-resistant prostate cancer. We have expanded these findings to evaluate the consequences for survival and metastases, to evaluate additional combinations that target these pathways, and to elucidate molecular pathways that are responsible for these drug responses *in vivo*.

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Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model

Carolyn Waugh Kinkade,[1] Mireia Castillo-Martin,[2,3] Anna Puzio-Kuter,[1,3] Jun Yan,[1,3] Thomas H. Foster,[4] Hui Gao,[5] Yvonne Sun,[5] Xuesong Ouyang,[5] William L. Gerald,[6,7] Carlos Cordon-Cardo,[1,2,3] and Cory Abate-Shen[1,2,3,5]

[1]Department of Urology, [2]Department of Pathology, and [3]Herbert Irving Comprehensive Cancer Center, Columbia University College of Physicians and Surgeons, New York, New York, USA. [4]Urologic University Clinic of Basel, Basel, Switzerland. [5]Center for Advanced Biotechnology and Medicine and The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, University of Medicine & Dentistry of New Jersey, Piscataway, New Jersey, USA. [6]Department of Pathology and [7]Department of Human Oncology and Pathogenesis, Memorial Sloan-Kettering Cancer Center, New York, New York, USA.

The AKT/mammalian target of rapamycin (AKT/mTOR) and ERK MAPK signaling pathways have been shown to cooperate in prostate cancer progression and the transition to androgen-independent disease. We have now tested the effects of combinatorial inhibition of these pathways on prostate tumorigenicity by performing preclinical studies using a genetically engineered mouse model of prostate cancer. We report here that combination therapy using rapamycin, an inhibitor of mTOR, and PD0325901, an inhibitor of MAPK kinase 1 (MEK; the kinase directly upstream of ERK), inhibited cell growth in cultured prostate cancer cell lines and tumor growth particularly for androgen-independent prostate tumors in the mouse model. We further showed that such inhibition leads to inhibition of proliferation and upregulated expression of the apoptotic regulator Bcl-2–interacting mediator of cell death (Bim). Furthermore, analyses of human prostate cancer tissue microarrays demonstrated that AKT/mTOR and ERK MAPK signaling pathways are often coordinately deregulated during prostate cancer progression in humans. We therefore propose that combination therapy targeting AKT/mTOR and ERK MAPK signaling pathways may be an effective treatment for patients with advanced prostate cancer, in particular those with hormone-refractory disease.

Introduction

Prostate cancer is one of the most common neoplasms, particularly among aging males in the United States. Like many adenocarcinomas, prostate tumors arise from preinvasive lesions, mainly prostatic intraepithelial neoplasia (PIN), which ultimately progress to adenocarcinoma and, in some cases, metastatic disease (1). Cancer progression, as well as all aspects of normal prostate differentiation, are critically dependent upon androgen receptor (AR) signaling (2).

While the prognosis for men diagnosed with early-stage disease has improved considerably in recent years, due to advances in the treatment of organ-confined prostate cancer, there are still few effective therapeutic options for advanced prostate cancer (3–5). The most common, namely abrogation of AR signaling via hormone deprivation therapy, is initially effective but ultimately leads to a hormone-refractory form of the disease, which is usually highly aggressive and frequently lethal. Although advances in chemotherapy have improved patient outcome (4–7), there remains a clear need for effective mechanism-based therapeutic approaches that can achieve long-term improvements in patient outcome.

Among the major signaling networks that have been implicated in advanced prostate cancer are the AKT/mammalian target of rapamycin (AKT/mTOR) and MAPK pathways. Indeed, deregulated expression and/or mutations of the phosphate and tensin homolog tumor suppressor gene (*PTEN*) occur with high frequency in prostate cancer, leading to aberrant activation of AKT kinase activity as well as its downstream effectors, including the mTOR signaling pathway (e.g., refs. 8–11). In addition, many prostate tumors display deregulated growth factor signaling, which may result in activation of MAPK kinase 1 (MEK) kinase and ultimately ERK MAP signaling (e.g., refs. 12, 13). Notably, previous studies have demonstrated that the AKT/mTOR and MAPK signaling pathways are alternatively and/or coordinately expressed in advanced prostate cancer and function cooperatively to promote tumor growth and the emergence of hormone-refractory disease (8, 11, 13–16). These observations formed the basis for our hypothesis that targeting these signaling pathways combinatorially may be effective for inhibiting tumorigenicity and androgen independence in prostate cancer.

In the current study, we have investigated the consequences of combinatorial inhibition of the AKT/mTOR and ERK MAPK signaling pathways by integrating preclinical studies in a genetically engineered mouse model, with analyses of the status of these pathways in human clinical specimens. We have employed the *Nkx3.1; Pten* mutant mouse model, which recapitulates many features of human prostate cancer (15, 17, 18). In particular, these mutant mice develop PIN, which progresses to adenocarcinoma with high penetrance (>90%) and with a highly reproducible time course of disease progression, while androgen deprivation leads to the emergence of hormone-refractory tumors (Figure 1A) (15, 17, 18). Furthermore, many key molecular pathways that are known to be altered in

Nonstandard abbreviations used: BPH, benign prostatic hyperplasia; CI, combination index; MEK, MAPK kinase 1; mTOR, mammalian target of rapamycin; S6K, p70 S6 kinase; PCa, prostate adenocarcinoma; PIN, prostatic intraepithelial neoplasia; TMA, tissue microarray.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* doi:10.1172/JCI34764.

human prostate cancer are also altered during cancer progression in these mice (19–21). Most relevant for the current study, *Nkx3.1; Pten* mutant mice display activation of AKT/mTOR and ERK MAPK signaling during prostate cancer progression in androgen-dependent and androgen-independent contexts (15). Therefore, we reasoned that these *Nkx3.1; Pten* mice should provide an excellent preclinical model to test the consequences of combinatorial targeting of AKT/mTOR and ERK MAPK signaling for prostate tumorigenesis.

We now report that combinatorial inhibition of the AKT/mTOR and ERK MAPK signaling pathways is highly effective for inhibition of prostate tumorigenicity in vivo, particularly for androgen-independent tumors. Furthermore, our analyses of the status of the PTEN/AKT/mTOR signaling pathway in human prostate tumors, as well as its correlation with activation of ERK MAPK signaling, confirm that these pathways are frequently deregulated in human prostate cancer and are, therefore, suitable targets for therapeutic intervention. We propose that combination therapy targeting the AKT/mTOR and ERK MAPK signaling pathways may be applicable to a broad spectrum of patients with advanced prostate cancer, particularly those with hormone-refractory disease, for which novel treatment options are urgently needed.

Results

Inhibition of AKT/mTOR and ERK MAPK signaling pathways with rapamycin and PD0325901. Based on previous studies showing that AKT/mTOR and ERK MAPK signaling pathways synergize to promote prostate tumorigenicity in human prostate cancer cell lines in culture as well as in *Nkx3.1; Pten* mutant mice in vivo (15, 16), we hypothesized that targeted therapy to combinatorially inhibit these signaling pathways would be effective for blocking prostate tumor growth. Therefore, we developed experimental paradigms to test the consequences of inhibiting these pathways individually or together in androgen-dependent and androgen-independent prostate tumors in the *Nkx3.1; Pten* mutant mice (Figure 1A).

In deciding upon the appropriate agents to test this hypothesis, our primary considerations were: (a) the accessibility/availability of relevant small-molecule inhibitors for these pathways; (b) the appropriateness of such agents for use in vivo as well as in cell culture; and (c) the feasibility of using such agents combinatorially. To achieve inhibition of ERK MAPK signaling, we opted to target MEK kinase, since it is directly upstream of ERK, which is considered to be its primary target (12). Notably, several MEK inhibitors are now available that have been shown to have potent anticancer growth properties, some of which are currently in clinical trials (22–24). For these studies, we used PD0325901 (from Pfizer), which is similar to its predecessor CI-1040 (24), albeit reported to have improved potency (22).

In lieu of AKT, effective inhibitors for which are still not widely available, we chose to target mTOR, since many of the downstream consequences of the AKT kinase are thought to be mediated through mTOR signaling (25, 26) and since components of the mTOR pathway have been shown to be activated in advanced prostate cancer (10). Moreover, unlike inhibitors of AKT, inhibitors of mTOR, namely rapamycin and its derivatives (e.g., CCI-779 from Wyeth and RAD001 from Novartis), are now readily available and considered to be promising anticancer agents (27). Notably, although their efficacy as single agents may be limited, rapamycin and related compounds are considered to be particularly suitable for use in combination therapy (e.g., refs. 27, 28). For these preclinical studies, we chose to use rapamycin (rather than one of the newer derivatives), since it is commercially available and therefore logistically more feasible to use in combination with PD0325901, which is obtained from Pfizer.

Although rapamycin and PD0325901 have been used as single agents in genetically engineered mice (24, 29–33), it was first necessary to define the optimal experimental parameters for their use in combination in vivo. To do so, we performed pilot studies in the *Nkx3.1; Pten* mutant mice to verify the optimal dosage as well as the appropriate dosing schedule for the agents delivered individually or in combination. For these and most subsequent studies, we used phosphorylation of S6 (p-S6), which is downstream of mTOR, as an indicator of mTOR pathway activity and phosphorylation (i.e., activation) of ERK MAPK as an indicator of MEK inhibition.

In general, we found that the optimal use of these agents in combination was similar to their previously reported use as single agents. In particular, we found that a single dose of rapamycin plus PD0325901 delivered to the *Nkx3.1; Pten* mutant mice individually or together resulted in the effective inhibition of their respective targets (i.e., p-S6 and p-ERK, respectively) for up to 24 hours (Figure 1B). Moreover, we found that the appropriate doses of rapamycin and PD0325901 needed to achieve effective inhibition of their respective targets, while resulting in limited toxicity or loss of body weight (see Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI34764DS1), were similar to those in published reports for their use as single agents. In particular, we found that i.p. delivery of 10 mg/kg of rapamycin resulted in significant inhibition of p-S6, and conversely that oral delivery of 20 mg/kg of PD0325901 resulted in effective inhibition of p-ERK, as evident by immunostaining and Western blot analyses of prostate tissues (Figure 1, B–J). Therefore, for these preclinical studies, we implemented a once-daily dosing schedule using 10 mg/kg of rapamycin and/or 20 mg/kg of PD0325901.

Rapamycin and PD0325901 synergize to inhibit prostate cell growth in culture. To quantify the individual and combinatorial effects of rapamycin and PD0325901, we performed studies in culture using mouse CASP prostate cancer cell lines, which were generated from primary tumors from the *Nkx3.1; Pten* mutant mice and include both androgen-responsive (CASP 2.1) and androgen-independent (CASP 1.1) lines (15, 19). First, we evaluated the IC_{50} of the single agents, which was 0.3 nM for rapamycin and 40 nM for PD0325901 in these CASP cells (Figure 2, A and B). In contrast, the IC_{50} of the agents in combination was shifted to 0.0018 nM for rapamycin and 11 nM for PD0325901 (Figure 2, C and D). To determine whether the shift in IC_{50} for the combination reflected their synergism in cell culture, we assessed their combination index (CI), which, as calculated based on the Chou-Talalay equation, provides a means of quantifying the differential sensitivity of agents in combination (34). The CI takes into account both potency (IC_{50}) and the shape of the dose-effect curve, such that a CI value of less than 1 indicates synergism, a CI value of 1 indicates an additive effect, while a value greater than 1 indicates antagonism. We found that rapamycin and PD0325901 displayed extremely low CIs (i.e., in the range of 0.03–0.1; Figure 2E), indicating their strong synergism in cell culture.

To gain initial insights regarding the mechanistic basis for the combinatorial effects of rapamycin and PD0325901, we investigated the expression status of various apoptotic regulatory proteins. We found that Bim (35, 36) was upregulated in response to the drug treatment, which is notable, since it has been shown to be upregulated by inhibitors of mTOR and MEK in other cell types

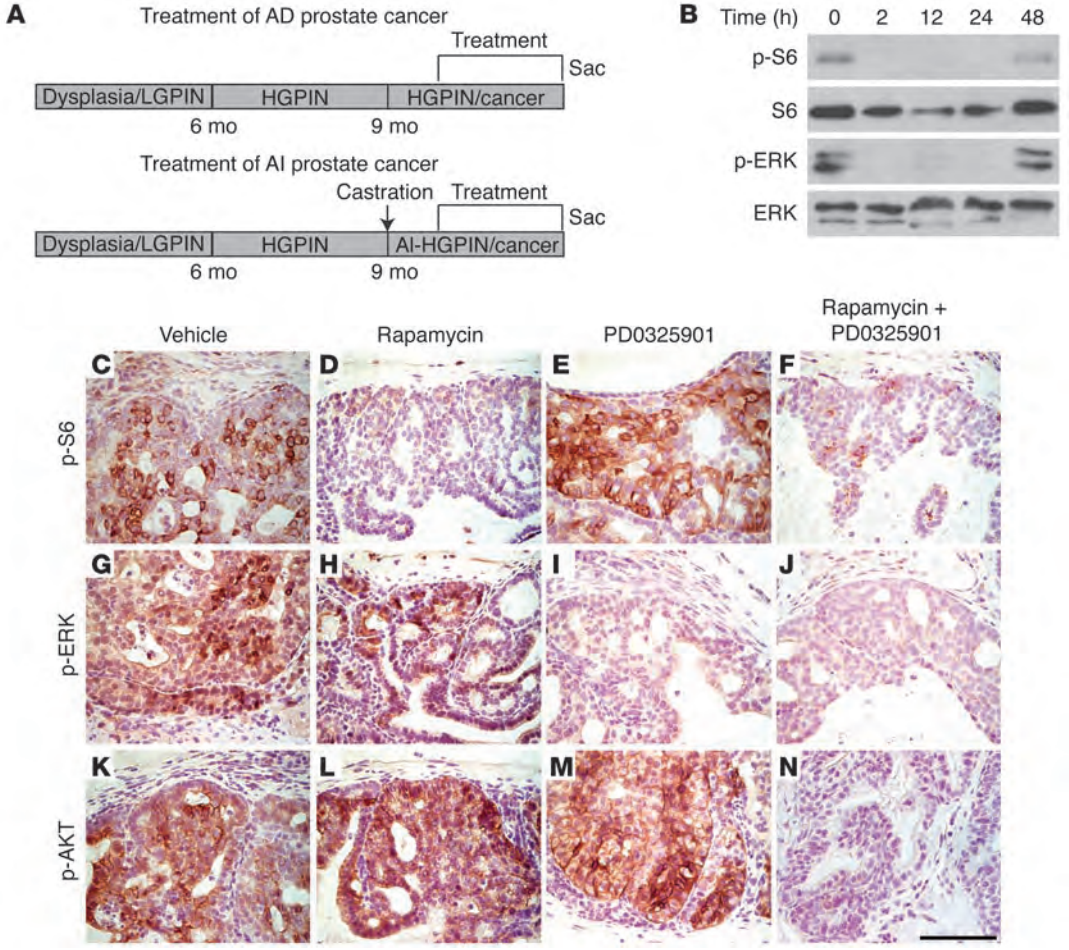

Figure 1

Inhibition of AKT/mTOR and ERK MAPK signaling pathways with rapamycin and PD0325901. (**A**) Diagram of the experimental strategy. mutant mice develop low-grade and high-grade PIN (LGPIN and HGPIN, respectively) and ultimately adenocarcinoma as a consequence of aging, as well as androgen independence following castration. The trial design entailed enrolling androgen-intact or androgen-ablated mutant (or control) mice at approximately 10 months of age randomly into groups that were treated with rapamycin and/or PD0325901 (or vehicle) for 21 days (5 days on/2 days off), after which the mice were sacrificed (Sac) for analyses of end points (i.e., histology, prostate weights, cellular proliferation, immunohistochemistry, and Western blot analyses; Figures 3–6 and Table 1). AD, androgen-dependent; AI, androgen-independent. (**B**) Rapamycin and PD0325901 inhibit their respective targets in the prostate for up to 24 hours. Western blot analyses were performed using protein extracts prepared from the dorsolateral prostate of mutant mice (10 months) treated with rapamycin plus PD0325901 for the times indicated. Each group had 3 mice; Western blot analyses were done with at least 2 independent mice in each group, and representative samples are shown. (**C**–**N**) Rapamycin and PD0325901 lead to inhibition of target proteins in mouse prostate tissues in vivo. Immunohistochemical analyses were performed using the indicated antibodies on sections from the anterior prostate of mutant mice (10 months; androgen-intact) treated with rapamycin and/or PD0325901 (or vehicle) as indicated for 1 week. Scale bar: 100 μm.

(e.g., ref. 37). Notably, while rapamycin and PD0325901 effectively inhibited their target pathways when used individually or in combination, upregulation of Bim expression was most marked when the drugs were used in combination (Figure 2F). Furthermore, we found that siRNAi-mediated knockdown of Bim resulted in a partial rescue of the consequences for cell survival in MTT assays, suggesting that the activity of these agents is mediated at least in part by upregulation of Bim (Figure 2G).

Collectively, these data suggest that rapamycin and PD0325901 act synergistically to promote cell death in culture, which reflects, at least in part, their combinatorial effects on the apoptotic regulator Bim. Furthermore, although our study is primarily focused on the efficacy of these agents in mutant mice, we have found that these agents also inhibit their respective pathways in human prostate cancer cells (Supplemental Figure 1), which suggests that these agents may have a similar benefit in human prostate cancer.

Preclinical analyses of combination therapy with rapamycin and PD0325901. Having demonstrated the combinatorial efficacy of rapamycin and PD0325901 for inhibiting their respective target pathways in prostate cells in vivo and in culture, as well as having established optimal experimental conditions for their combined use in vivo, we next investigated their potential antitumor effects in *Nkx3.1; Pten* mutant mice. In particular, we performed preclinical studies to compare the consequences of these agents for: (a) treatment of androgen-dependent prostate cancer (i.e., in mice that were androgen-intact); and (b) treatment of androgen-inde-

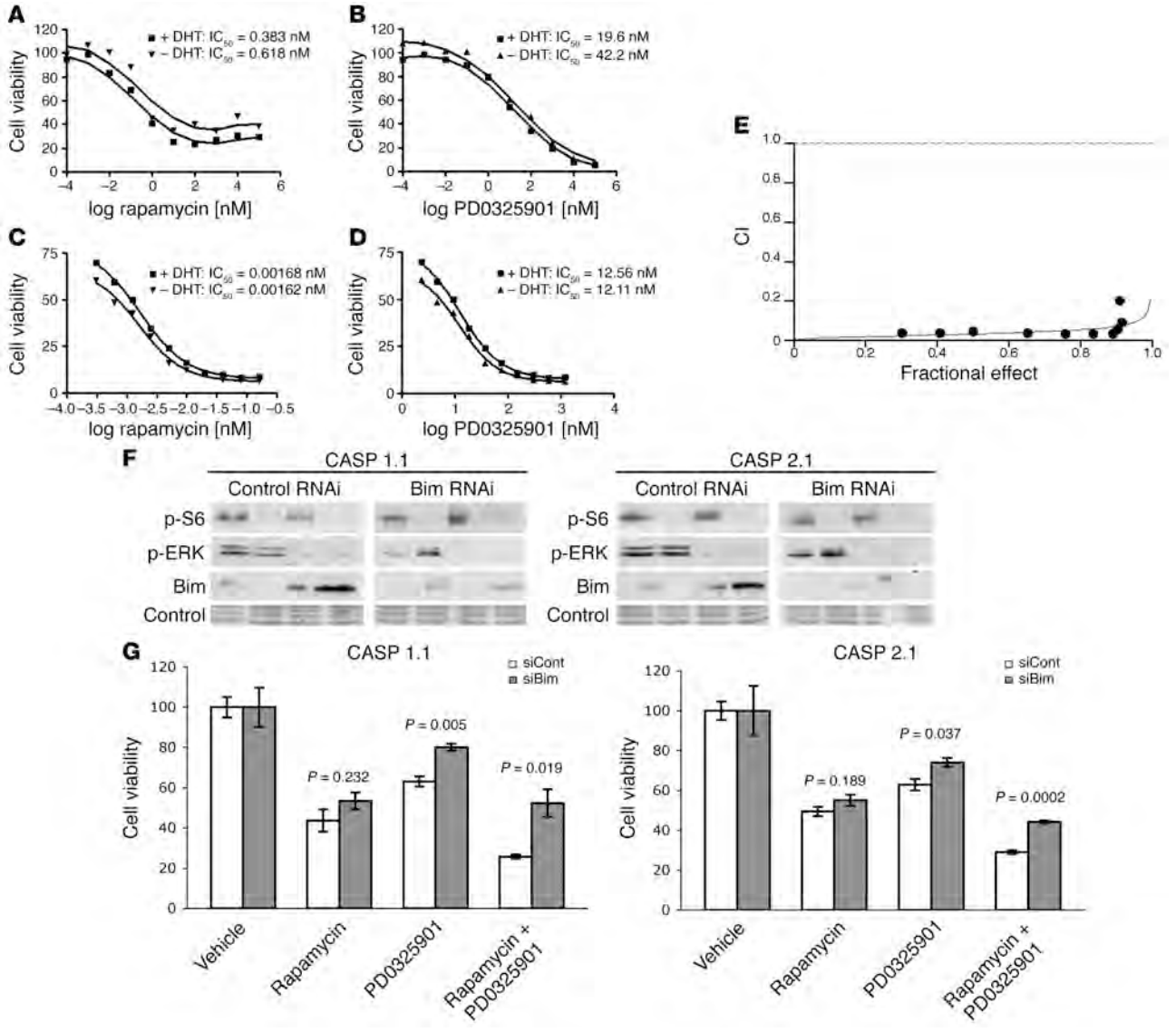

Figure 2

Rapamycin and PD0325901 display strong synergism in cell culture. (**A**–**D**) Analyses of IC_{50} plots for the single agents (**A** and **B**) and the combination agents (**C** and **D**). (**E**) Graphic representation of the CI for rapamycin and PD0325901. These data are shown for CASP 1.1 cells; similar results were obtained for CASP 2.1 cells (not shown). Note that CI values were well below 0.1, indicating strong synergism of the drugs. (**F** and **G**) Bim is upregulated in response to drug treatment. CASP 1.1 or CASP 2.1 cells were transfected with a control or Bim siRNAi, followed by treatment with vehicle or the indicated compounds in the medium for 48 hours. (**F**) Western blot analyses done on whole cell extracts using the indicated antibodies or a control for protein loading (Ponceau S staining). (**G**) Results of MTT assays, indicating the enhanced cell survival in the drug-treated cells following treatment with Bim siRNAi. The values compare the control (siCont) and the Bim siRNAi (siBim) in each group. Data are expressed as mean ± SEM.

pendent prostate cancer (i.e., in mice that had been castrated and had developed hormone-refractory tumors) (Figure 1A).

We used *Nkx3.1; Pten* mutant mice (i.e., *Nkx3.1*$^{+/-}$*Pten*$^{+/-}$ or *Nkx3.1*$^{-/-}$*Pten*$^{+/-}$) or wild-type littermates (i.e., *Nkx3.1*$^{+/+}$*Pten*$^{+/+}$) at 10–12 months, by which age the mutant mice display virtually complete penetrance of high-grade PIN with associated adenocarcinoma, as well as complete penetrance of hormone-refractory tumors following surgical castration (Figure 1A) (15, 17, 18). Cohort groups were composed of mutant (or control) mice randomly assigned to receive vehicle alone, single agents (rapamycin or PD0325901), or the combination therapy (rapamycin plus PD0325901) (Table 1 and Supplemental Table 1). Agents were provided for a period of 3 weeks using a dosage schedule of once daily for 5 days, with 2 days off to allow the mice to recover. Endpoint analyses included semiquantitative histological, immunohistochemical, and Western blot analyses, as well as quantitative assessments of prostate tissue weights and proliferation index (Figures 3–5; summarized in Table 1 and Supplemental Table 1); notably, we assessed outcome based on analyses of the combination of these various endpoints rather than any individual parameter.

To augment these studies in the whole animal, we performed complementary studies using a tissue recombination model in

Table 1
Data summary

Experimental group	No. of mice	Tumor weight		Proliferation	
		Fold change	*P*	Fold change	*P*
Paradigm 1: Treatment of AD cancer					
Vehicle	6	NA	NA	NA	NA
Rapamycin	4	2.25	0.066	2.05	0.175
PD0325901	4	0.88	0.453	1.66	0.197
Rapamycin + PD0325901	5	1.73	0.111	2.44	0.165
Paradigm 2: Treatment of AI cancer					
Vehicle	9	NA	NA	NA	NA
Rapamycin	5	1.38	0.062	2.13	0.032
PD0325901	6	1.30	0.041	2.74	0.008
Rapamycin + PD0325901	10	2.18	0.0002	14.64	0.0001
Paradigm 3: Adjuvant therapy of AI cancer					
Vehicle	6	NA	NA	NA	NA
Rapamycin	8	2.18	0.022	2.56	0.071
PD0325901	8	3.00	0.010	3.21	0.022
Rapamycin + PD0325901	12	3.32	0.012	3.35	0.035

AD, androgen-dependent; AI, androgen-independent.

which prostate epithelium from *Nkx3.1; Pten* mutant (or wild-type) mice is combined with wild-type rat mesenchyme and grown under the kidney capsule of androgen-intact or androgen-ablated *nude* male hosts (15) (Figure 6 and Supplemental Table 1). This strategy complements studies done in the whole animal (i.e., in the *Nkx3.1; Pten* mutant mice) in 2 important respects. First, it enables evaluation of the efficacy of combination therapy in vivo for the intended target cells (i.e., the prostate epithelial cells), as opposed to the target tissue (i.e., intact prostate). Second, because of the nature of how the tissue recombinants are made, tumor growth is relatively uniform (and therefore more easily quantified) in the tissue recombination model, in contrast to the intrinsically more heterogenous tumor growth that occurs in the whole animal. However, since preclinical studies are inherently more meaningful in the context of the whole animal with an intact immune response, studies done in the *Nkx3.1; Pten* mutant mice and the tissue recombination model are complementary, not redundant.

Combination therapy with rapamycin and PD0325901 is potently antitumorigenetic for androgen-independent prostate cancer. To test the efficacy of rapamycin and PD0325901 for the treatment of androgen-dependent prostate cancer, we performed preclinical studies in androgen-intact *Nkx3.1; Pten* mutant mice as well as in tissue recombinants grown in androgen-intact *nude* male hosts using the experimental conditions outlined above (Figure 3, Figure 5, A–D, Figure 6, A–H, Table 1, and Supplemental Table 1). We found that, whether delivered individually or in combination, rapamycin and PD0325901 effectively inhibited their respective target pathways in prostate tissues, as evidenced by immunohistochemistry and Western blot analysis for p-S6 and p-ERK (Figure 3, F–M, and Figure 5D). Notably, in combination, these agents resulted in a more striking inhibition of their target proteins, as well as inhibition of p-AKT (Figure 3, B–Q, and Figure 5D).

Nonetheless, both the single agents and the combination were only modestly effective for inhibiting prostate tumorigenicity in this context. For example, the combination therapy produced a modest, albeit not statistically significant, reduction in prostate weight in the whole animal (1.7-fold; $P = 0.111$) and proliferation rate (2.4-fold; $P = 0.165$) (Figure 3, R–U, Figure 5, B and C, and Table 1). However, in the tissue recombinant model, the drug combination did result in a significant inhibition of tumor size (2.6-fold; $P = 0.033$) (Figure 6, A–H), which likely reflects the uniformity of tumor growth in the tissue recombinants relative to the whole animal. Therefore, although rapamycin and PD0325901 effectively inhibit mTOR and ERK MAPK signaling in androgen-dependent prostate cancer, they do not optimally inhibit tumor growth in this context.

In striking contrast, rapamycin and PD0325901 were highly effective for inhibiting androgen-independent tumor growth in the *Nkx3.1; Pten* mutant mice (Figure 4, Figure 5, E and F, and Figure 6, I–P). In particular, both the single agents and the combination therapy resulted in inhibition of their respective target proteins, p-S6 and p-ERK, although as above, the combination produced a more profound inhibition of these proteins as well as the additional inhibition of p-AKT (Figure 4, B–Q, and Figure 5H). Moreover, while the single agents each resulted in a statistically significant reduction in tumor size and proliferation (see Table 1 and Figure 5, F and G), the combination therapy produced a profound abrogation of the histological phenotype, as well as a significant reduction in tumor weights (2.2-fold; $P = 0.0002$) and cellular proliferation (14.7-fold; $P = 0.0001$) (Figure 4, B–E and R–U, Figure 5, F and G, and Table 1). Moreover, the efficacy of this drug combination for androgen-independent prostate cancer was recapitulated in the tissue recombination model of the disease (3.2-fold; $P = 0.007$) (Figure 6, I–P). These findings demonstrate that combination therapy targeting the AKT/mTOR and ERK MAPK signaling pathways is highly effective for the treatment of hormone-refractory prostate cancer in a preclinical model of the disease.

Considering the profound effects of this combination therapy for inhibiting androgen-independent prostate cancer, we investigated whether this drug combination might also be effective in an adjuvant therapy model for hormone-refractory prostate cancer.

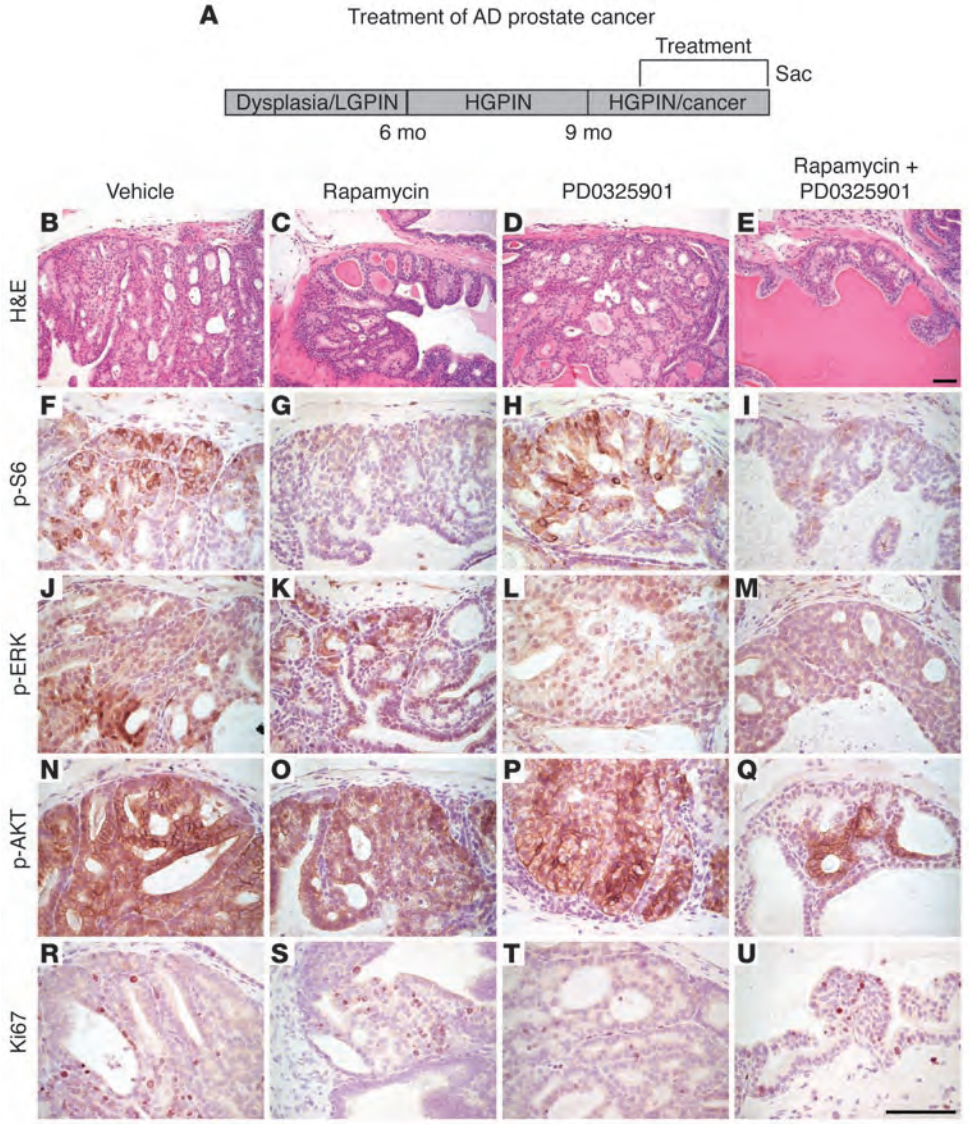

Figure 3
Modest efficacy of rapamycin and PD0325901 for treatment of androgen-dependent prostate cancer. (**A**) Diagram of the experimental strategy, as per Figure 1. (**B**–**U**) Representative tissue sections from the mutant mice treated with the single agent or combination of agents, showing histological phenotype (H&E) and immunostaining for the antibodies indicated. H&E analyses were performed on all experimental mice in each group; immunohistochemistry was done on a minimum of 4 animals from each group; representative data are shown. Scale bars: 100 μm.

Specifically, rapamycin and PD0325901 were provided for a period of 3 weeks directly following androgen ablation of the *Nkx3.1; Pten* mutant mice or tissue recombinants grown in the *nude* mice (Figure 7 and Table 1). In this context, the drug combination was highly effective for inhibiting prostate tumor growth, as evidenced by the striking effects on the histological phenotype, as well as the significant reduction in the prostate weight (3.3-fold; $P = 0.012$) and cellular proliferation (3.4-fold; $P = 0.035$) in the *Nkx3.1; Pten* mutant mice and in the analogous tissue recombination model (2.2-fold; $P = 0.018$) (Figure 7). Therefore, this combination therapy may be promising for adjuvant therapy of hormone-refractory prostate cancer when used in combination with androgen deprivation therapy.

AKT/mTOR and ERK MAPK signaling pathways are coordinately regulated in human prostate cancer. Extrapolating from these preclinical studies in the mutant mouse model, our findings suggest that combination therapy targeting the AKT/mTOR and ERK MAPK signaling pathways may be beneficial for treatment of patients with hormone-refractory prostate cancer. Notably, the potential to translate these findings from mice to human prostate cancer is particularly promising considering that the combination is effective for inhibiting their target pathways in human prostate cancer cells (Supplemental Figure 1) and since the single agents (or related agents) are already being used in clinical trials (22, 27).

However, the efficacy and expected versatility of the combination therapy depends, in part, upon: (a) the frequency with which these signaling pathways are deregulated individually and combinatorially in human prostate cancer, particularly in patients with advanced disease; and (b) the ease of identifying patients who have primary tumors in which these pathways are deregulated and, therefore, would be appropriate candidates for targeted therapy.

While these issues have been partially addressed in previous studies that have examined the status of individual components of these signaling pathways in human prostate cancer (e.g., refs. 8–11, 13), we have now performed a comprehensive analysis in primary tumors and metastases from human prostate cancer patients to evaluate the coordinate expression of multiple components of the PTEN/AKT/mTOR signaling pathway, as well as their status relative to ERK MAPK signaling (Figure 8 and Table 2). In particular, we performed analyses using 2 independent cohorts of patients assembled on 2 independent tissue microarrays (TMAs). This com-

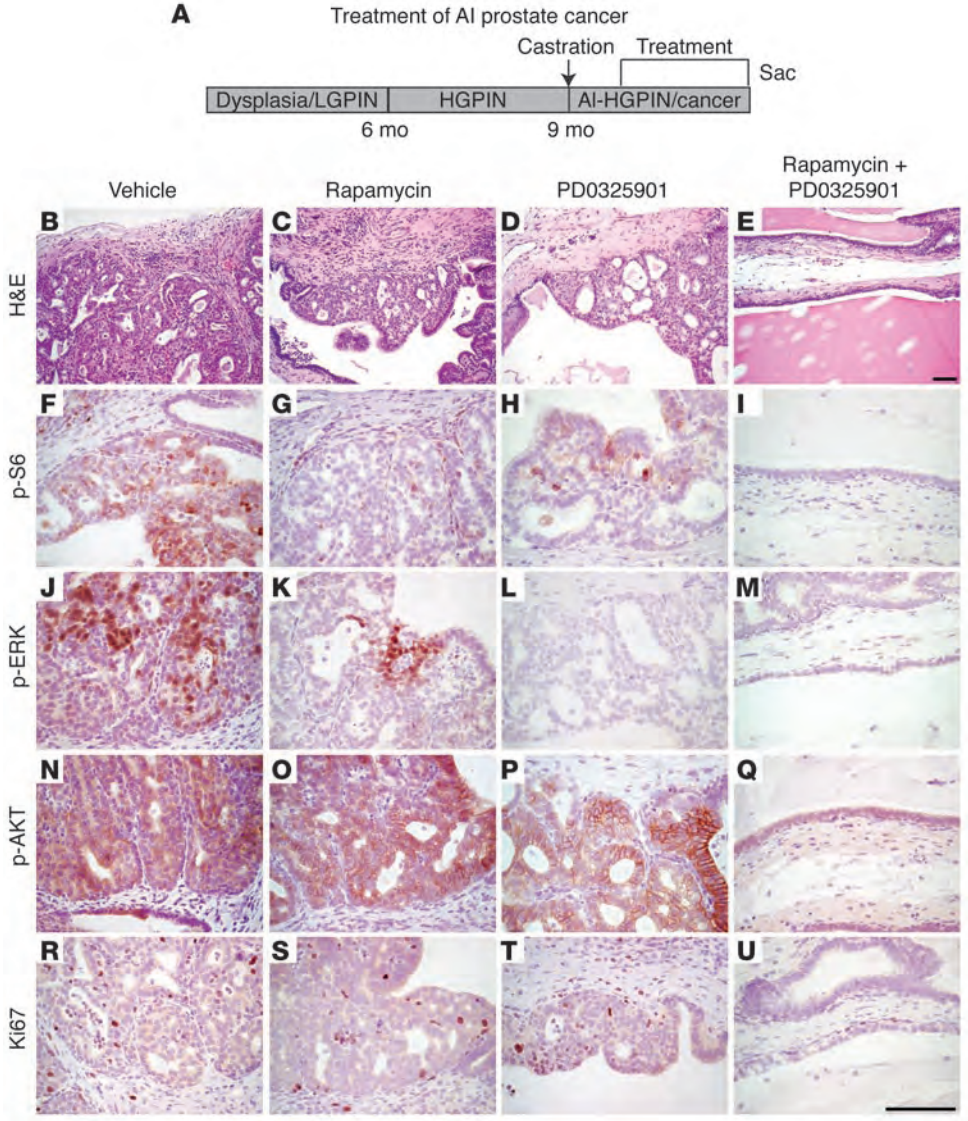

Figure 4
Profound efficacy for prostate histology following treatment of androgen-independent prostate cancer with rapamycin and PD0325901. (**A**) Diagram of the experimental strategy, as per Figure 1. (**B**–**U**) Representative tissue sections from the androgen-ablated mutant mice treated with the single or combination of agents, showing histological phenotype (H&E) and immunostaining for the antibodies indicated. In these and all subsequent experiments, H&E analysis was performed on all experimental mice in each group; immunohistochemistry was done on a minimum of 4 animals from each group; representative data are shown. Scale bars: 100 μm.

prehensive series enabled us to examine the coordinate expression of several components of the PTEN/AKT/mTOR pathway, as well as ERK MAPK, in specimens from patients with benign prostatic hyperplasia (BPH); PIN; prostate adenocarcinoma (PCa), including those with low Gleason (≤6) or high Gleason (>7) scores; hormone-refractory cancer; and metastases. In particular, we examined the expression levels of PTEN as well as the activation (phosphorylation) of AKT, mTOR, and p70 S6 kinase (S6K) relative to each other as well as to activation of ERK MAPK.

The first TMA comprised 70 samples, including 25 cases of BPH, 7 of PIN, 19 of low Gleason score cancer, and 19 of high Gleason score cancer (Table 2). We found that PTEN expression was markedly reduced with high frequency (59% cases) during prostate cancer progression, as determined by comparing BPH, PIN, and PCa ($P = 0.05$; Figure 8, A–C; PIN data not shown). We further found that the expression levels of both p-AKT and p-mTOR were significantly elevated in prostate cancer compared with BPH and PIN ($P = 0.001$; Figure 8, D–I; PIN data not shown). Similarly, p-S6K expression was significantly higher in prostate cancer compared with BPH and PIN ($P < 0.0001$; Figure 8, J–L; PIN data not shown). Notably, when activated, these proteins were frequently activated in the same patient samples and also coincident with reduced expression of PTEN (Figure 8, C, F, I, L, and O, and Tables 2–4), indicating that the pathway rather than the individual components are coordinately deregulated.

To further validate these results, as well as the potential clinical implications of identifying an altered PTEN/AKT/mTOR signaling pathway in prostate cancer, we used a second TMA composed of an independent cohort of prostate cancer patients. This TMA represented a large, well-characterized group of 535 patients, including 65 cases of BPH, 78 of PIN, 181 of organ-confined cancer, 120 of hormone-refractory cancer, and 91 of metastatic PCa. We found that decreased levels of PTEN were significantly associated with disease progression, mainly when comparing primary and metastatic prostate cancer ($P = 0.001$). Similarly, increased levels of p-AKT ($P = 0.02$), p-mTOR ($P = 0.02$), and p-S6K ($P = 0.03$) were also found to be significantly associated with tumor progression. Furthermore, these proteins were often activated together and with reduced expression of PTEN, again indicative of deregulation of the signaling pathway.

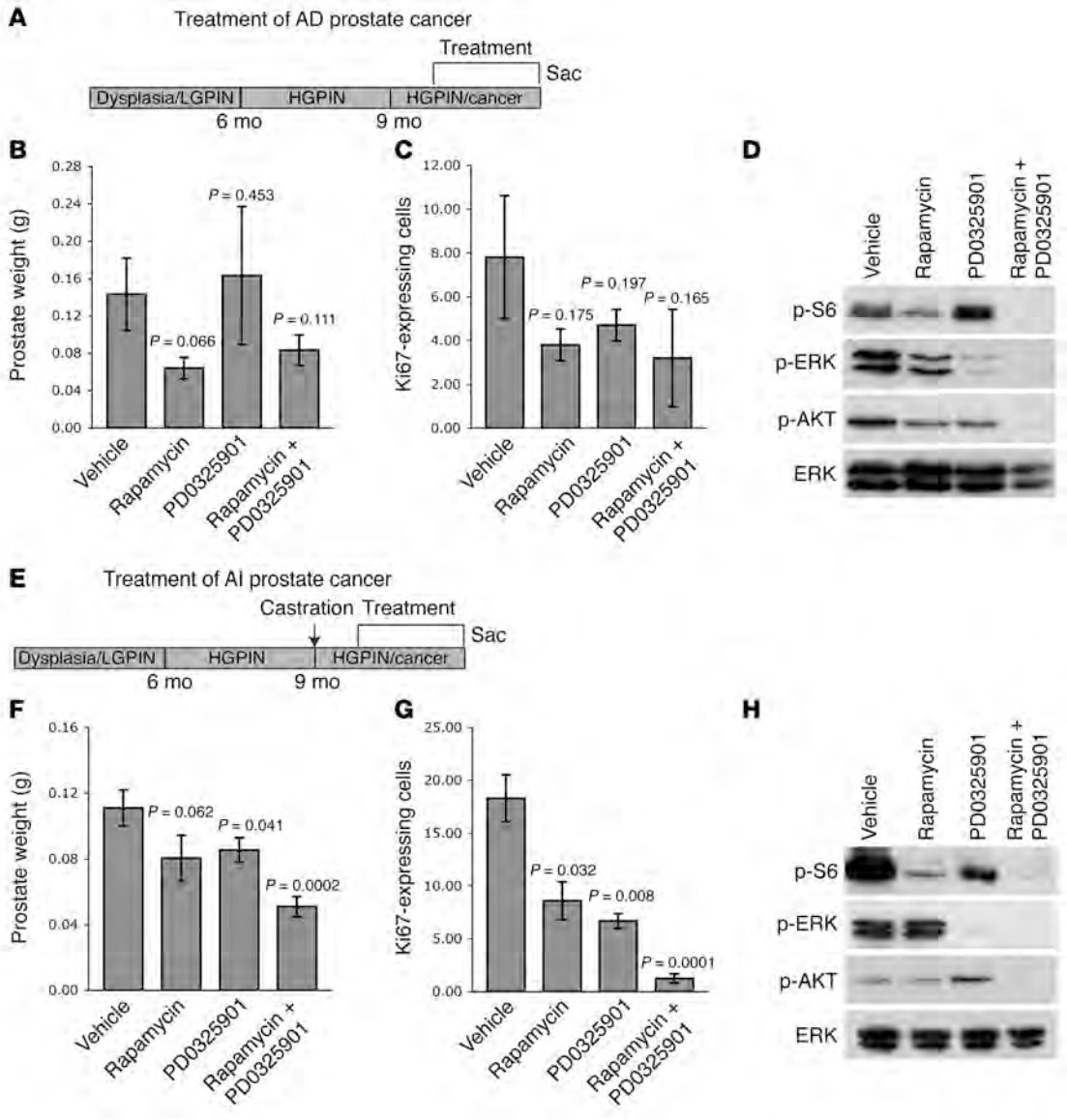

Figure 5
Combination therapy is antitumorigenic for treatment of androgen-independent prostate cancer in mutant mice. Comparison of the consequences of single agent versus combination therapy for treatment of androgen-dependent (**A**–**D**) or androgen-independent (**E**–**H**) mutant mice. (**A** and **E**) Diagram of the experimental strategy, as per Figure 1. (**B** and **F**) Prostate tissue weights, showing mean ± SEM, with value indicated. (**C** and **G**) Percentage of proliferating cells as determined by Ki67 staining, showing mean ± SEM, with value indicated. (**D** and **H**) Western blots of protein extracts from prostate tissues following treatment. Western blotting was done using a minimum of 3 independent mice in each group; representative data are shown; total ERK is shown as a control for protein loading.

Finally, using this first TMA, we examined the expression of ERK MAPK in human prostate tumor samples, compared with the deregulation of the PTEN/AKT/mTOR signaling pathway (Tables 2–4). Immunohistochemical analysis of p-ERK expression showed that it was focally expressed and at low levels in BPH samples and PIN lesions (Figure 8M; PIN data not shown). However, p-ERK was highly expressed in the majority of cancer samples, and increased levels of ERK MAPK were significantly associated with tumor progression in PIN and low-grade tumors versus high-grade lesions ($P = 0.008$; Figure 8, M–O). Furthermore, when compared with deregulated expression of the PTEN/AKT/mTOR signaling pathway, we found that 21% of patients with prostate cancer displayed deregulated expression of all components of the PTEN/AKT/mTOR signaling pathway as well as ERK MAPK, while 42% of these patients displayed deregulated expression of 1 or more components of the PTEN/AKT/mTOR signaling pathway as well as ERK MAPK (Table 4).

In conclusion, the PTEN/AKT/mTOR signaling pathway is frequently altered in prostate cancer progression and often coincidentally with activation of ERK MAPK signaling. These data support the concept that a substantial number of patients with advanced prostate cancer (conservatively at least 20%) may benefit from combination therapy targeting the AKT/mTOR and ERK MAPK signaling pathways. Furthermore, since the relevant activated target

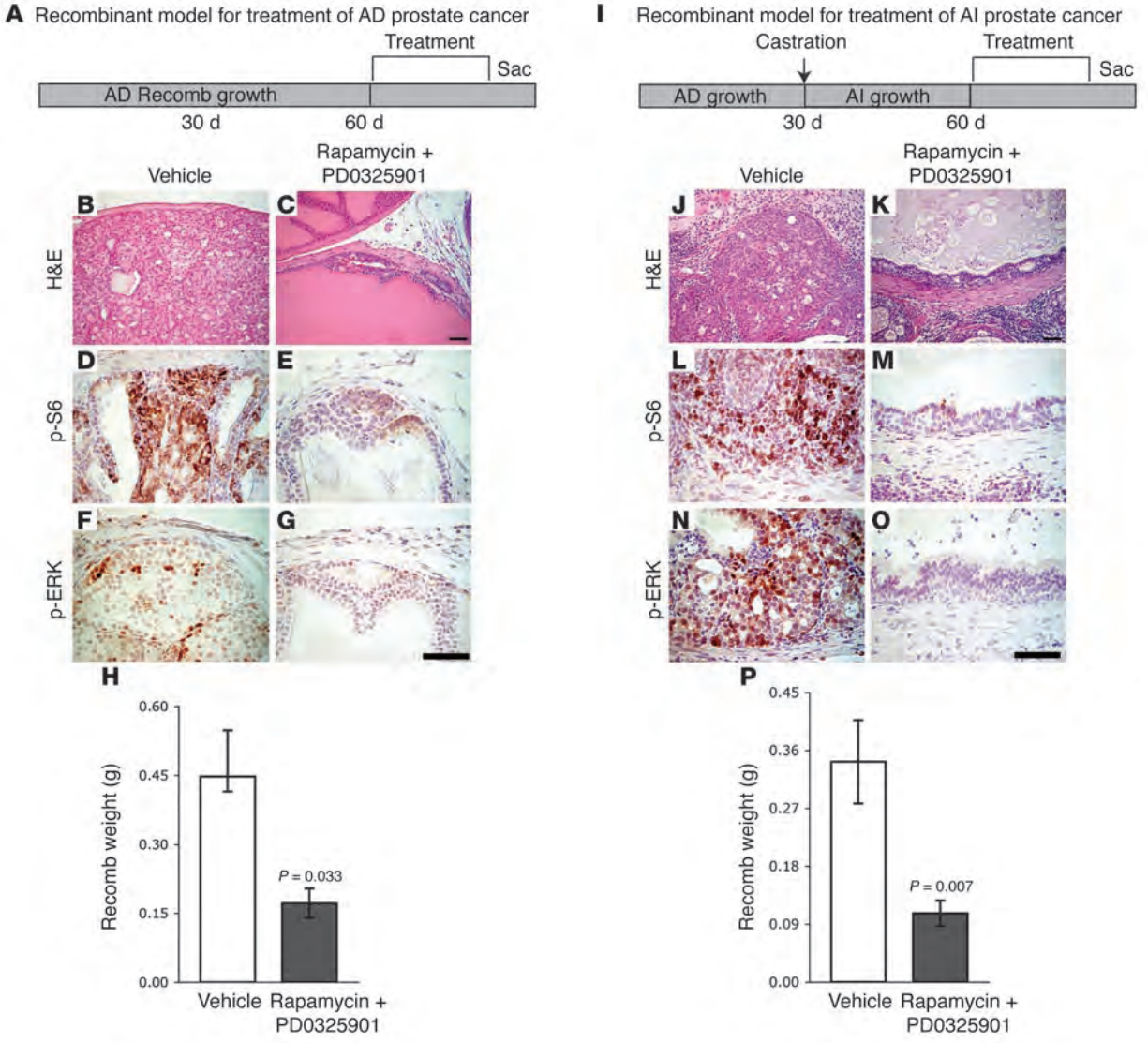

Figure 6

Combination therapy is antitumorigenic in tissue recombination models from the mutant mice. (**A** and **I**) Diagram of the experimental strategy. Tissue recombinants were made using prostate epithelium from mutant or wild-type mice and rat embryonic mesenchyme and grown in mice for 1 month. Following 1 month of growth, the mice were either left intact (i.e., to model treatment of androgen-dependent prostate cancer) or castrated (i.e., to model treatment of androgen-independent prostate cancer). Mice received rapamycin and/or PD0325901 (or vehicle) for 21 days (5 days on/2 days off), following which the mice were sacrificed for analyses of end points (i.e., histology, prostate weights, and immunohistochemistry). (**B**–**G** and **J**–**O**) Representative tissue sections from the tissue recombinants made from the prostate epithelium showing histological phenotype (H&E) and immunostaining for p-S6 and p-ERK, as indicated. Scale bars: 100 μm. (**H** and **P**) Weights of the tissue graphs, showing mean ± SEM, with value indicated. Recomb, recombinant.

proteins can be readily detected in primary tumors from patient samples, it should be feasible to identify patients that are most likely to respond to treatment, namely those that display deregulation of PTEN/AKT/mTOR and/or ERK MAPK signaling.

Discussion

In our previous investigations of the molecular mechanisms underlying prostate cancer progression, we found that the AKT/mTOR and ERK MAPK signaling pathways function cooperatively to promote prostate tumorigenicity and androgen independence (15). Based on these findings, we had hypothesized that combinatorial inhibition of these pathways might be effective for the treatment of prostate cancer. We now demonstrate that inhibition of these signaling pathways acts combinatorially to suppress pathway activation and inhibit tumor growth and cellular proliferation in androgen-independent prostate cancer in *Nkx3.1; Pten* mutant mice. Furthermore, analyses of human prostate tumor specimens support the idea that AKT/mTOR and ERK MAPK signaling pathways are frequently activated in prostate tumors, readily detected in advanced human prostate cancer, and are suitable targets for intervention in patients with the disease. We propose that combination therapy targeting the AKT/mTOR and the ERK MAPK signaling pathways may be effective for treatment of a broad spectrum of patients with advanced prostate cancer, particularly those with hormone-refractory disease.

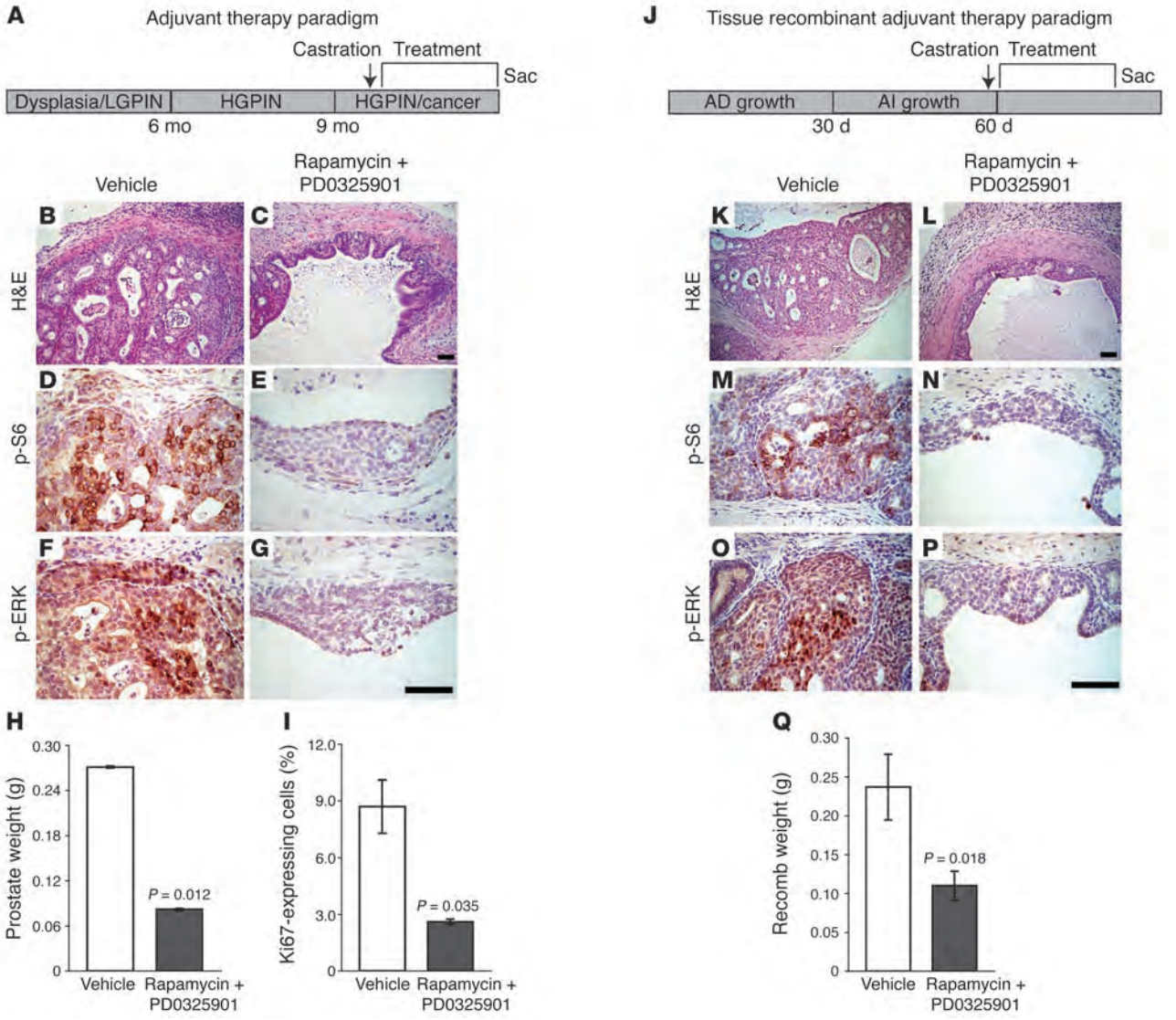

Figure 7

Combination therapy is antitumorigenic in adjuvant therapy of hormone-refractory prostate cancer. (**A**–**I**) Studies in the whole animal. (**A**) Diagram of the experimental strategy. Strategy is similar to that in Figure 1; however, unlike the androgen-independent treatment group, mutant mice were castrated immediately prior to receiving the rapamycin and/or PD0325901 (or vehicle) treatment. (**B**–**G**) Representative tissue sections from the mutant mice showing histological phenotype (H&E) and immunostaining for p-S6 and p-ERK, as indicated. (**H**) Prostate tissue weights, showing mean ± SEM, with value indicated. (**I**) Percentage of proliferating cells as determined by Ki67 staining, showing mean ± SEM, with value indicated. (**J**–**Q**) Studies done in a complementary tissue recombination model. (**J**) Diagram of the experimental strategy. (**K**–**P**) Representative tissue sections from the tissue recombinants made from the prostate epithelium showing histological phenotype (H&E) and immunostaining for p-S6K and p-ERK, as indicated. (**Q**) Weights of the tissue graphs, showing mean ± SEM, with value indicated. Scale bars: 100 μm.

Although conventional chemotherapeutic approaches have been proven effective in many contexts, now including prostate cancer (5, 6, 38), it has become increasingly evident that ultimately the most effective and safest long-term treatment options for cancer patients will be achieved by targeting the specific networks that are deregulated in tumors, as exemplified by the now classic example of Gleevec for the treatment of leukemia (39). However, targeted therapies using single agents often lead to resistance, as also exemplified by Gleevec (40). It has been proposed that tumor resistance can be circumvented, at least in part, using agents in combination that can simultaneously target multiple pathways — the idea being that coordinate suppression of multiple pathways may minimize the chances of developing resistant tumor cells.

This general strategy of combination therapy requires knowledge of relevant signaling networks that are deregulated in particular cancers, an understanding of how their coordinate and/or cooperative activities contribute to tumorigenesis, and relevant in vivo model systems to test the functional consequences of therapeutic targeting of such pathways for tumorigenesis. Indeed, while informative analyses in tumor cells in culture may provide mechanistic insights, studies done in the context of the tumor microenvironment in the whole

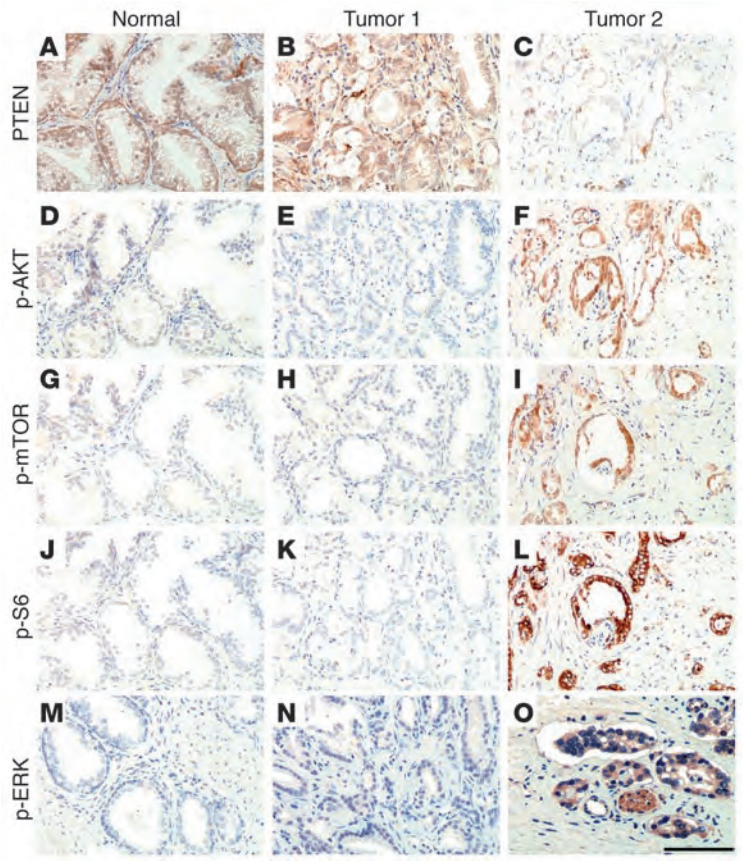

Figure 8
AKT/mTOR pathway activation is associated with human prostate cancer progression and correlated with activation of ERK MAPK. (**A**–**L**) Expression of PTEN and components on the AKT/mTOR signaling pathway in human normal and primary prostate cancer samples. Representative adjacent sections from the specimens used for the TMAs show staining for the indicated proteins. Shown are examples of benign prostatic tissue (Normal), a tumor without activation of the PTEN/AKT/mTOR pathway (Tumor 1), and a tumor with activation of this pathway (Tumor 2). Note that the expression of PTEN is inversely correlated with expression of p-AKT, p-mTOR, and p-S6. (**M**–**O**) Expression of p-ERK activation on semiadjacent sections of the same specimens. Note that, in these samples, p-ERK activation is well correlated with activation of components of the AKT/mTOR signaling pathway. Scale bar: 100 μm.

organism are likely to provide more robust strategies for predicting the efficacy of combinatorial inhibition for tumor growth in vivo. In this regard, analyses of relevant genetically engineered mutant mouse models may be informative for predicting both the pathways that may be effectively targeted and the therapeutic efficacy of single and combinatorial inhibition of such pathways (41–44).

Indeed, our current analyses exemplify the value of this general approach, as we have effectively used the *Nkx3.1; Pten* mutant mouse model both to define the functional significance of the AKT/mTOR and ERK MAPK signaling pathways for prostate tumorigenicity and to demonstrate the profound antitumorigenic consequences of combinatorial inhibition of these pathways for prostate tumorigenicity. Importantly, our findings show that the combination therapy is considerably more effective than the single agents with respect to their antitumorigenic activity, which appears to reflect in part their effects on the expression of the apoptotic regulator Bim. Further insights regarding the mechanisms underlying the striking synergism of these pathways is provided by the accompanying article by Carracedo and colleagues, who report that these pathways are modulated by a negative feedback loop (45).

More generally, an important consideration when using combination targeted therapy is that, more so than for conventional chemotherapy, the efficacy of the treatment for any single individual is likely to depend upon whether the specific pathways are deregulated in tumors from such individuals. Thus, accurate evaluation of the efficacy of targeted therapies is likely to depend on knowledge of the status of the relevant pathways prior to the onset of treatment, since clinical success or failure may well depend upon the status of such pathways. Indeed, our findings showing that activation of relevant target proteins in the AKT/mTOR and/or ERK MAPK signaling pathways can be readily detected in primary tumors from human prostate cancer patients (see Figure 8) demonstrate the feasibility of this approach and provides an effective screening tool for both enrolling patients in clinical trials and evaluating the outcome of such trials.

Table 2
Summary of TMA data

Pathology	No. of cases	PTEN (neg)	p-AKT (pos)	p-mTOR (pos)	p-S6 (pos)	p-ERK (pos)
BPH	25	0%	28%	20%	8%	40%
PIN	7	0%	43%	14%	14%	43%
Low-grade cancer	19	37%	63%	42%	32%	74%
High-grade cancer	19	22%	79%	53%	53%	68%

neg, negative; pos, positive.

Table 3
Correlation of staining on TMA

Correlation	p-AKT	p-mTOR	p-S6	p-ERK
p-AKT	ND	67%	67%	73%
p-mTOR	100%	ND	70%	60%
p-S6	100%	70%	ND	60%
p-ERK	85%	43%	57%	ND

ND, not determined.

Furthermore, our analyses of pathway activation in human prostate cancer have shown that a large percentage of advanced tumors display activation of AKT/mTOR and/or ERK MAPK signaling networks and predict that a large percentage of prostate cancer patients (>20%) will be appropriate candidates for treatment with agents that target these signaling pathways. Extrapolating from preclinical trials in the *Nkx3.1; Pten* mutant mice to the design of relevant clinical trials, we infer that combination therapy targeting AKT/mTOR and ERK MAPK signaling should be optimal for patients with hormone-refractory disease. Conceivably, combination therapy may be effective when used in conjunction with chemotherapy, as there are now several ongoing trials combining chemotherapy with mechanism-based approaches (4).

Notably, at present, the reason(s) for the enhanced efficacy of this combination therapy in prostate cancer in conditions of limiting androgens are unclear. Presumably, this reflects, at least in part, the fact that these pathways may be preferentially activated in hormone-refractory prostate cancer (8, 11, 13–15). However, it seems likely that there may be mechanistic bases that are dependent on the tissue context, such as potential variations in the activity levels of these kinases in the presence or absence of androgens (46). Regardless, our findings showing the efficacy of this combination treatment when provided simultaneously with androgen ablation (Figure 7) raise the exciting possibility that combination therapy targeting AKT/mTOR and MEK/ERK MAPK signaling may even be effective in an adjuvant therapy model for hormone-refractory disease.

In summary, our findings as well as recent work by Stommel and colleagues and Shah and colleagues (47, 48) highlight the value of pathway-targeted combination therapy to achieve maximal blockade of signaling pathways for cancer treatment. Furthermore, our study demonstrates the value of pursuing hypothesis-based preclinical trials in genetically engineered mutant mice that share relevant features with the human cancers that they represent.

Methods

Mouse models. All experiments using mice were approved by the Institutional Review Board of Columbia University Medical College. The *Nkx3.1; Pten* compound mutant mice have been described previously (15, 17, 18). Preclinical studies were done in a mixed strain background (C57BL/6 × 129S/V) using cohorts assembled from littermates of wild-type ($Nkx3.1^{+/+}Pten^{+/+}$) and mutant ($Nkx3.1^{-/-}Pten^{+/-}$ or $Nkx3.1^{+/-}Pten^{+/-}$) mice at 10–12 months of age that had been housed together and subjected to identical environmental conditions. Mice were randomly assigned to the androgen-intact group or the androgen-independent group; the latter were surgically castrated to remove the testes and epididymis, which is the source of endogenous androgens. Tissue recombinants were made using prostate epithelium from mutant or wild-type mice and rat embryonic mesenchyme and grown in androgen-intact or androgen-deprived (castrated) *nude* male hosts, as described previously (15, 17).

Preclinical trial design and analyses. Rapamycin was purchased from LC Laboratories (catalog R-5000, lots ASW-105 and ASW-109); PD0325901 was a generous gift from Pfizer (Batch U). Rapamycin was dissolved in 100% ethanol to make a working stock of 25 mg/ml and then diluted to 1.25 mg/ml in a solution of 5.2% Tween-80, 5.2% PEG400 in sterile water and delivered i.p. at 10 mg/kg. PD0325901 was dissolved in 0.05% hydroxy-propyl-methylcellulose, 0.02% Tween-80 in sterile water to make a working stock of 1.5 mg/ml and delivered via oral gavage at 20 mg/kg. Agents (or vehicle) were delivered for 5 consecutive days, with 2 days off for a period of 3 weeks. Mice were weighed daily and observed for signs of distress following dosing. Notably, none of the treatment paradigms resulted in appreciable weight loss (i.e., greater than 10%) in the mutant mice (Supplemental Table 1).

At the conclusion of the study, mice were sacrificed, prostate tissues were collected and photographed, and the wet weights of the prostate tissues were determined. For the tissue recombinant models, the graphs were removed from the kidney prior to weighing. The prostatic lobes (anterior, dorsolateral, and ventral) were collected individually and bilaterally; one lobe was fixed in formalin and paraffin-embedded for histology and immunohistochemistry and the other snap frozen in liquid nitrogen for Western blotting. All analyses were done using both the anterior and dorsolateral prostatic lobes; histological and immunohistochemical analyses are shown for the anterior prostate and Western blot analyses for the dorsolateral gland.

Immunohistochemical studies, Western blot analyses, and semiquantitative analyses of the histological phenotype were done as described previously (15, 17, 18). Briefly, for grading of the histological phenotype, a minimum of 4 random sections from different locations in the prostate tissues were examined for each experimental animal. The phenotype was scored based on analyses of the multiple sections in each animal plus the analyses of all animals, following which a representative tissue section was selected to illustrate the phenotype. Analyses of the immunohistochemical studies were done using 2–3 independent sections from each of 4 experimental animals in each group. For quantification of proliferating cells, slides were immunostained with Ki67, and cells were counted as described previously (49) in a minimum of 10 independent sections from 5 independent mice (i.e., 50 sections), with results expressed as the percentage of Ki67-labeled epithelium relative to total epithelium, visualized using hematoxylin.

Antibodies used in this study were: p-p44/42 MAPK (Thr202/Tyr204) antibody (catalog 9101 or IHC preferred antibody 4376; 1:200), p44/42 MAPK antibody (catalog 9102), p-AKT (Ser473) antibody (catalog 9271 or IHC preferred antibody 3787; 1:50), and p-S6 Ribosomal Protein (Ser235/236) antibody (catalog 2211; 1:250), all from Cell Signaling Technology; Bim antibody (catalog 2065; 1:500) from ProSci Inc.; and NCL-Ki67p (1:1,500) from Novocastra (Leica Microsystems).

Cell culture analyses. Cytotoxicity assays were performed using the CASP 2.1 or 1.1 line (15, 19). Exponentially growing cells were seeded into 96-well plates at 800 cells/well in 100 μl growth medium in the presence or absence of dihydrotestosterone. Rapamycin or PD0325901 was serially diluted in media, and the cells were maintained at 37°C for 96 hours. Cell density was determined following addition of 20 μl MTS reagent (Promega) by measurement of the absorbance at 490-nm wavelength. The IC_{50} was deter-

Table 4
Activation of pathways in patient specimens

Activated	Cases (%)
Neither the PTEN pathway nor ERK[A]	8
PTEN pathway and ERK[B]	21
PTEN pathway (partial) and ERK	42

[A]e.g., tumor 1 in Figure 8. [B]e.g., tumor 2 in Figure 8.

mined using the software SoftMax (Molecular Devices). For combination drug treatment, rapamycin plus PD0325901 was diluted in the growth medium at a fixed ratio of doses (IC_{50} versus IC_{50}). The CI was calculated based on the Chou-Talalay equation (34) using CalcuSyn software (Biosoft). For Bim-knockdown studies, CASP 1.1 and 2.1 cells were transfected with 50 nM of control or Bim siRNA (Santa Cruz Biotechnology Inc.), according to the manufacturer's protocol. One day after transfection, cells were treated with rapamycin and/or PD0325901 for 48 hours. Cytotoxity assays were performed using a Cell Growth Determination Kit, MTT Based (Sigma-Aldrich), according to the manufacturer's protocol.

Human TMAs. The human TMAs were made using samples from prostatectomy specimens obtained from the Molecular Pathology Tumor Bank of Columbia University. All studies of human tissue were approved by the Institutional Review Board of Columbia University Medical Center. To construct the TMAs, sections of normal prostate and tumor tissue that were embedded in paraffin and stained with H&E were reviewed to identify viable, morphologically representative areas of the specimen from which needle core samples could be taken. From each specimen, triplicate tissue cores with diameters of 0.6 mm were punched and arrayed onto a recipient paraffin block using a precision instrument (Beecher Instruments). Five-micrometer sections of these TMA blocks were stained with H&E or used for immunohistochemical analysis. Prostate tumor samples included PIN and PCa, which was classified as having either a low Gleason (≤6) or high Gleason (>7) score.

Two TMAs were used for the present study. The first included 70 prostate samples: 25 BPH cases, 7 PIN lesions, and 38 PCas (19 displayed a low Gleason score and 19 a high Gleason score). Each tissue was represented by 3 independent cores in each TMA. The second TMA included 535 prostate samples: 65 corresponded to BPH, 78 to PIN, and 392 to PCas (181 cases corresponded to organ-confined prostate cancer, 120 cases to hormone-refractory prostate cancer, and 91 cases to metastatic prostate cancer). Seven consecutive sections of the 2 TMAs were cut and stained with H&E (verification of histopathology), used as a negative control, or stained with immunohistochemical markers.

Immunohistochemical analyses were performed following the standard avidin-biotin immunoperoxidase staining procedure. Briefly, TMA slides were deparaffinized and then submitted to antigen retrieval by steamer treatment for 15 minutes in 10 mM citrate buffer at pH 6.0, followed by primary antibody incubation overnight at 4°C. Then slides were incubated with biotinylated anti-rabbit or anti-mouse immunoglobulins at a 1:1,000 dilution for 30 minutes (Vector Laboratories Inc.) followed by avidin-biotin peroxidase complexes at a 1:25 dilution (Vector Laboratories Inc.) for 30 minutes. Diaminobenzidine was used as the chromogen and hematoxylin as a nuclear counterstain. Primary antibodies used were against PTEN (Ab-6, mouse clone 6H2.1; Neomarkers, Thermo Scientific; 1:50), p-AKT (Ser473, rabbit polyclonal; Cell Signaling Technology; 1:50), p-mTOR (Ser 2448, rabbit clone 49F9; Cell Signaling Technology; 1:50), S6K (rabbit polyclonal; Cell Signaling Technology; 1:75), and p44/42 MAPK (rabbit clone 137F5; Cell Signaling Technology; 1:100).

The immunoreactivity for each antibody was scored according to the percentage of cells displaying a positive immunostaining profile (from undetectable [0%] to homogeneous expression [100%]) and the intensity of the staining (0, 1+, 2+, and 3+). Average values of the 3 representative cores from each arrayed sample were used for statistical analyses. Expression values were displayed as mean values accompanied by 95% confidence intervals and range. The relationship between immunohistochemical results and clinical parameters was analyzed using the nonparametric Wilcoxon–Mann-Whitney *U* tests. A *P* value of less than 0.05 was considered statistically significant.

Acknowledgments

We are indebted to Pfizer for sharing the PD325901 compound. We thank Mitchell Benson, Edward Gelmann, Daniel Petrylak, and Michel Shen for comments on the manuscript. This work was supported in part by National Cancer Institute (NCI) grants to C. Abate-Shen (U01-CA84294 and R01-CA115717), as well as funding from the T.J. Martell Foundation. C. Cordon-Cardo and his laboratory are, in part, supported by the NCI SPORE CA92629 grant in Prostate Cancer and grants from The T.J. Martell Foundation and the Sands Family Foundation. A. Puzio-Kuter was supported by 1F31CA110625-01 Ruth L. Kirschstein National Research Service Award. T.H. Foster was supported, in part, by the Swiss National Science Foundation (81BS-69441) and the Swiss Urologic Association.

Received for publication December 14, 2007, and accepted in revised form June 25, 2008.

Address correspondence to: Cory Abate-Shen, Columbia University College of Physicians and Surgeons, Herbert Irving Comprehensive Cancer Center, 1130 St. Nicholas Avenue, Room 217A, New York, New York 10032, USA. Phone: (212) 851-4731; Fax: (212) 851-4572; E-mail: cabateshen@columbia.edu.

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Molecular genetics of prostate cancer: new prospects for old challenges

Michael M. Shen and Cory Abate-Shen

Genes Dev. 2010 24: 1967-2000
Access the most recent version at doi:10.1101/gad.1965810

Molecular genetics of prostate cancer: new prospects for old challenges

Michael M. Shen[1,2,3,6] and Cory Abate-Shen[3,4,5,7]

[1]Department of Medicine, Columbia University Medical Center, New York, New York 10032, USA; [2]Department of Genetics and Development, Columbia University Medical Center, New York, New York 10032, USA; [3]Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, New York 10032, USA; [4]Department of Urology, Columbia University Medical Center, New York, New York 10032, USA; [5]Department of Pathology and Cell Biology, Columbia University Medical Center, New York, New York 10032, USA

Despite much recent progress, prostate cancer continues to represent a major cause of cancer-related mortality and morbidity in men. Since early studies on the role of the androgen receptor that led to the advent of androgen deprivation therapy in the 1940s, there has long been intensive interest in the basic mechanisms underlying prostate cancer initiation and progression, as well as the potential to target these processes for therapeutic intervention. Here, we present an overview of major themes in prostate cancer research, focusing on current knowledge of principal events in cancer initiation and progression. We discuss recent advances, including new insights into the mechanisms of castration resistance, identification of stem cells and tumor-initiating cells, and development of mouse models for preclinical evaluation of novel therapeutics. Overall, we highlight the tremendous research progress made in recent years, and underscore the challenges that lie ahead.

In 2009, there were ~192,280 new cases of prostate cancer reported and 27,360 related deaths in the United States (American Cancer Society 2009). Although the age-adjusted rate of cancer deaths has decreased steadily in the past 10 years, prostate cancer remains the second leading cause of cancer death in men. Since we last reviewed this topic 10 years ago (Abate-Shen and Shen 2000), there have been numerous advances in basic research on prostate cancer initiation and progression, as well as new clinical advances that have improved patient outcome. Below, we review the principal features of prostate cancer, highlighting key molecular events of initiation and progression and major targets for clinical intervention. When feasible, we cited primary references for the key findings discussed, particularly those published within the past 10 years.

[*Keywords*: Androgen receptor; castration resistance; mouse models; prostate cancer; stem cells]
Correspondence.
[6]E-MAIL mshen@columbia.edu; FAX (212) 851-4572.
[7]E-MAIL cabateshen@columbia.edu; FAX (212) 851-4572.
Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.1965810.

Major clinical challenges in prostate cancer

Prostate cancer has been recognized as a clinical entity since antiquity, when it was first described by the ancient Egyptians, while surgical procedures to remove the prostate were developed >100 years ago (Capasso 2005). However, the availability of a highly accessible blood test for prostate-specific antigen (PSA) has revolutionized the diagnosis of prostate cancer over the past three decades. PSA is a kallikrein-related serine protease that is produced in normal prostate secretions, but is released into the blood as a consequence of disruption of normal prostate architecture (Lilja et al. 2008).

Men that have elevated PSA levels typically undergo biopsy to assess the potential presence of prostate cancer. Following biopsy, histopathological grading of prostate tissue is performed by Gleason scoring, which classifies tumors from 1 to 5 (most to least differentiated) based on their most prevalent architecture, and assigns a combined score that is the sum of the two most common patterns (Mellinger et al. 1967; Epstein 2010). Patients are also diagnosed by the status of their primary tumors, from organ-confined to fully invasive (T1–4), with or without lymph node involvement (N0 or 1), and the presence and degree of distant metastases (M0 and 1a–c) (Ohori et al. 1994). If prostate cancer is diagnosed, conventional treatment regimens include surgical excision of the prostate (radical prostatectomy), or irradiation through external beam therapy or implantation of radioactive "seeds" (brachytherapy). In the case of advanced cancer, these regimens are usually followed or substituted with androgen deprivation therapy, which initially will reduce tumor burden and/or circulating PSA to low or undetectable levels, but ultimately the disease will recur in most cases.

At present, there are several major clinical challenges associated with this conventional paradigm for prostate cancer diagnosis and treatment. Each of these significantly impacts the effective management of prostate cancer, and is the subject of investigations in basic research on prostate tumor biology.

Distinguishing indolent vs. aggressive disease

Recent changes in recommendations that now suggest later and less frequent PSA screenings highlight a major clinical challenge for prostate cancer diagnosis and treatment (Wolf et al. 2010). These new recommendations were proposed because the widespread use of PSA testing has led to a vast increase in the diagnosis of patients with clinically localized low Gleason grade carcinomas that may not require treatment, since their tumors are relatively indolent. In particular, patients with a Gleason pattern of 3 or less almost never relapse after local therapy, and very likely can be managed conservatively with "watchful waiting"; nonetheless, a small fraction of these tumors will progress rapidly and require immediate treatment (Albertsen et al. 2005; Eggener et al. 2007; Lu-Yao et al. 2009).

Consequently, a major clinical challenge is posed by the current inability to readily distinguish indolent from aggressive tumors in prostate cancer patients who present with low Gleason grade tumors upon biopsy (Sartor et al. 2008). The absence of this prognostic information has led to a significant "overtreatment" of patients who would otherwise require only conservative management. Thus, the impact of treatment on prostate cancer survival is small, most likely because overdiagnosis and overtreatment dilutes the benefits of therapy for those who require intervention. This prognostic challenge could be addressed by better understanding of the molecular basis of cancer initiation, which should ultimately lead to the identification of biomarkers that distinguish between indolent and aggressive forms of prostate cancer. At present, however, available panels of molecular biomarkers do not provide greater prognostic significance than Gleason grade determination (True et al. 2006).

Castration-resistant prostate cancer

Circulating androgens are essential for normal prostate development as well as the onset of prostate cancer through their interactions with the androgen receptor (AR). As shown by Huggins and colleagues in the 1940s (Huggins and Hodges 1941), removal of testicular androgens by surgical or chemical castration will lead to regression of prostate tumors. However, androgen depletion is usually associated with the recurrence of prostate cancer, as monitored by rising PSA levels, and this recurrent disease is termed "castration resistant." (The term "castration resistance" has generally replaced "androgen independence" in usage, as it has become apparent that advanced prostate cancer remains dependent on AR function, as discussed below.) Unfortunately, castration-resistant prostate cancer has been essentially untreatable, with the most effective standard chemotherapeutic regimens resulting in a mean increase in survival time of 2 mo (Petrylak et al. 2004; Tannock et al. 2004). Therefore, a second major clinical challenge that could be significantly impacted by basic research in prostate cancer biology is the elucidation of pathways of castration resistance, which could lead to the identification of new therapeutic approaches.

Bone tropism of prostate cancer metastasis

A third major clinical challenge corresponds to the propensity for advanced prostate cancer to metastasize to bone, which is primarily responsible for its effect on patient morbidity as well as mortality. Thus, unlike other epithelial tumors that occasionally metastasize to bone, metastatic prostate cancer almost invariably metastasizes to bone, and furthermore displays characteristic osteoblastic rather than osteolytic lesions (Logothetis and Lin 2005). Despite the clinical relevance of bone metastasis, the molecular mechanisms that underlie the bone tropism of prostate cancer are not well understood. This gap in knowledge is due in part to difficulties in obtaining metastatic tissue from patients, as well as to difficulties in generating mouse models that display bone metastasis.

At present, relatively little is known about the molecular mechanisms underlying the bone tropism of prostate cancer metastasis. Experimental models for investigation of bone metastases are limited to a small range of xenograft models that typically rely on intracardiac or intratibial injection of highly transformed tumor cells to induce metastases (Corey et al. 2002; Singh and Figg 2005). To date, despite the availability of genetically engineered mouse models that display secondary metastases, there is no autochthonous model that reliably generates bone metastases at an appreciable frequency.

Development of the prostate gland

Anatomy and histology

In men, the prostate gland is a walnut-sized tissue surrounding the urethra at the base of the bladder, and produces important components of the seminal fluid. Although the adult prostate lacks discernible lobular structure, the classic work of McNeal (1969, 1981, 1988) defined the human prostate as having a zonal architecture, corresponding to central, periurethral transition, and peripheral zones, together with an anterior fibromuscular stroma (Timms 2008). Importantly, the outermost peripheral zone occupies the most volume, and harbors the majority of prostate carcinomas. In contrast, benign prostatic hyperplasia (BPH), a common nonmalignant condition found in older men, arises from the transition zone.

Unlike the human prostate, the mouse prostate consists of multiple lobes that have distinct patterns of ductal branching, histological appearance, gene expression, and secretory protein expression (Cunha et al. 1987). These correspond to the ventral, lateral, dorsal, and anterior lobes, with the dorsal and lateral lobes often combined as the dorsolateral lobe for analysis. Although it is sometimes asserted that the mouse dorsolateral lobe is most analogous to the human peripheral zone, particularly with respect to prostate cancer, there is no consensus agreement among pathologists to support this conclusion (Shappell et al. 2004). However, analyses of gene expression profiling data support the idea that the dorsolateral lobe is most similar to the peripheral zone of the human prostate (Berquin et al. 2005).

At the histological level, both the mouse and human prostate contain a pseudostratified epithelium with three differentiated epithelial cell types: luminal, basal, and neuroendocrine (CS Foster et al. 2002; van Leenders and Schalken 2003; Hudson 2004; Shappell et al. 2004; Peehl 2005). The luminal epithelial cells form a continuous layer of polarized columnar cells that produce protein secretions and express characteristic markers such as cytokeratins 8 and 18, as well as high levels of AR. Basal cells are located beneath the luminal epithelium, and express p63 and the high-molecular-weight cytokeratins 5 and 14, but express AR at low or undetectable levels. Finally, neuroendocrine cells are rare cells of unknown function that express endocrine markers such as chromogranin A and synaptophysin, but are AR-negative.

Epithelial–mesenchymal interactions

The prostate is an endodermal tissue that arises during late embryogenesis through ductal budding from the anterior urogenital sinus epithelium. Formation of the prostate is an inductive event that requires reciprocal interactions between the urogenital sinus mesenchyme and epithelium, and is dependent on testicular androgen synthesis. The fundamental parameters of these epithelial–mesenchymal interactions were defined in classical tissue recombination studies by Cunha and colleagues (Cunha et al. 1987; Cunha 2008). These studies demonstrated that an AR-dependent signal from the urogenital mesenchyme is required for prostate formation, while AR is not initially required in the urogenital epithelium for prostate organogenesis, but is subsequently necessary for epithelial differentiation and secretory protein expression. Thus, androgens act indirectly on the urogenital mesenchyme to mediate prostate induction. These findings have been subsequently confirmed by conditional gene targeting of AR in the prostate epithelium (Wu et al. 2007).

More recently, molecular analyses have implicated several developmental signaling pathways in mediating epithelial–mesenchymal interactions during prostate organogenesis, including the Wnt, fibroblast growth factor (FGF), and Hedgehog pathways (Marker et al. 2003; Prins and Putz 2008). For example, ligands and inhibitors for the canonical Wnt/β-catenin as well as noncanonical pathways are expressed in both epithelial and mesenchymal compartments during early prostate organogenesis (Pritchard and Nelson 2008), and abrogation of noncanonical Wnt5a signaling leads to defects in ductal morphogenesis (Huang et al. 2009). In addition, the FGF pathway is clearly required for prostate formation, as null mutants for the mesenchymally expressed *Fgf10* mostly lack prostate budding (Donjacour et al. 2003), while conditional deletion of *Fgfr2*, which encodes the receptor for FGF10, or the downstream signaling component *Frs2α* in prostate epithelium results in defects in branching morphogenesis (Lin et al. 2007; Zhang et al. 2008). Finally, the Hedgehog signaling pathway is also involved in prostate formation, as the Shh ligand is expressed in urogenital epithelium; the downstream components *Smo*, *Ptc1*, and *Gli1* are expressed in urogenital mesenchyme (Lamm et al. 2002; Freestone et al. 2003; Berman et al. 2004); and loss of Shh pathway activity results in loss of prostate formation and/or defective ductal branching (Podlasek et al. 1999; Freestone et al. 2003; Berman et al. 2004). However, it remains unclear whether these phenotypes are mediated directly through redundant ligands functioning through the Hedgehog pathway (Doles et al. 2006), or indirectly through a reduction in androgen signaling (Freestone et al. 2003; Berman et al. 2004).

Natural history of prostate cancer

Latent and clinical cancer

Prostate cancer is generally regarded as multifocal, since primary tumors often contain multiple independent histologic foci of cancer that are often genetically distinct (Aihara et al. 1994; Bostwick et al. 1998; Macintosh et al. 1998; Mehra et al. 2007a; Clark et al. 2008). In contrast, despite the phenotypic heterogeneity of metastatic prostate cancer (Shah et al. 2004), molecular and cytogenetic analyses show that multiple metastases in the same patient are clonally related, indicating that advanced prostate cancer is monoclonal (Mehra et al. 2008; Liu et al. 2009). These findings suggest that metastatic prostate cancer may arise from the selective advantage of individual clones during cancer progression; however, this process of clonal evolution may also represent the consequence of therapeutic interventions such as androgen deprivation, which may differentially target cells of varying malignant potential.

The heterogeneity of prostate cancer is potentially relevant for understanding the distinction between latent and clinical disease, and the strong correlation between prostate cancer progression and aging (Fig. 1). Although prostate cancer is a disease of older men, studies of prostate specimens from healthy men in their 20s to 40s show the frequent presence of histologic foci of prostate cancer (Yatani et al. 1989; Sakr et al. 1994; Shiraishi et al. 1994), suggesting that cancer initiation has already taken place at a relatively early age. Combined with the evidence that prostate cancer is multifocal, it appears that the prostate gland can be the site of multiple neoplastic transformation events, many of which give rise only to latent prostate cancer that does not progress to clinically detectable disease. It is conceivable that clinical prostate cancer initiates from a different pathogenic program than latent prostate cancer. Alternatively, most latent prostate cancer foci may not undergo critical activating events that lead to clinical disease, or may remain under active suppression sufficient to maintain these foci in a subclinical state. As discussed above, the advent of PSA screening has led to a vast increase in the diagnoses of prostate cancer, many of which presumably represent latent or indolent forms of the disease that at present are difficult to distinguish from cancers that will become more aggressive; this highlights the critical need for improved molecular markers and/or other approaches to augment the histological assessment of prostate cancer for more effective diagnosis and management.

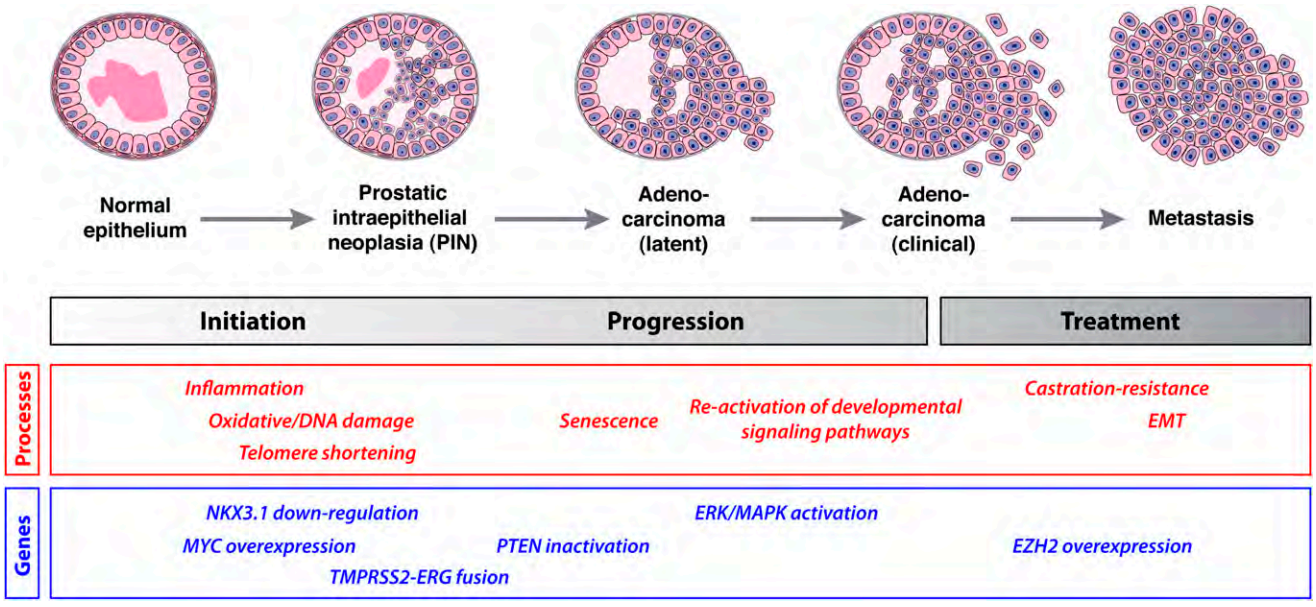

Figure 1. Progression pathway for human prostate cancer. Stages of progression are shown, together with molecular processes and genes/pathways that are likely to be significant at each stage. Adapted from Abate-Shen and Shen (2000).

Prostatic intraepithelial neoplasia (PIN) and prostate cancer

It is widely accepted that PIN represents a precursor for prostate cancer, although this relationship has not been demonstrated conclusively (Bostwick 1989; DeMarzo et al. 2003). PIN is generally characterized at the histological level by the appearance of luminal epithelial hyperplasia, reduction in basal cells, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia, and nuclear atypia; in addition, high-grade PIN lesions generally display marked elevation of cellular proliferation markers (Bostwick 1989; Shappell et al. 2004). In contrast with prostate cancer, however, basal cells are reduced in number in PIN, but are not absent.

Although human prostate cancer displays significant phenotypic heterogeneity, >95% of prostate cancers are classified pathologically as adenocarcinoma, which has a strikingly luminal phenotype (Fig. 2). In biopsy specimens, prostate adenocarcinoma diagnosis can be confirmed by the absence of immunostaining using p63 and cytokeratin 5/14 antibodies, both of which detect basal cells (Humphrey 2007; Grisanzio and Signoretti 2008). In addition, a diagnosis of prostate cancer is supported by elevated immunostaining for α-methylacyl-CoA racemase (AMACR), a luminal marker that is overexpressed in carcinoma (Luo et al. 2002; Jiang et al. 2005; Humphrey 2007). Similarly, prostate cancer arising in many mouse models displays a relatively luminal phenotype (Kim et al. 2002d; X Ma et al. 2005). However, the overt histological appearance of prostate carcinoma in most genetically engineered mouse models often differs from that of typical human prostate cancer (Fig. 2).

Subtypes of prostate cancer

A notable difference between prostate cancer and other epithelial tumors, such as breast cancer, is the lack of distinguishable histopathological subtypes that differ in their prognosis or treatment response. The vast majority of prostate cancers correspond to acinar adenocarcinomas that express AR, while other categories of prostate cancer—such as ductal adenocarcinoma, mucinous carcinoma, and signet ring carcinoma—are extremely rare

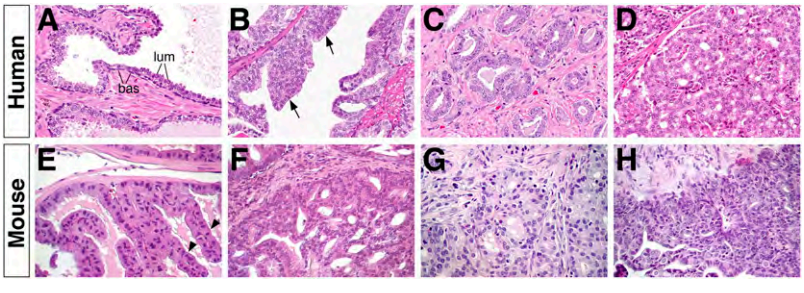

Figure 2. Histopathology of human and mouse prostate cancer. (*A–D*) Hematoxylin-eosin-stained sections of human prostate. (*A*) Benign normal tissue, with representative basal (bas) and luminal (lum) cells indicated. (*B*) PIN; arrows indicate regions of hyperplastic epithelium. (*C*) Well-differentiated adenocarcinoma. (*D*) Poorly differentiated adenocarcinoma. (*E–H*) Hematoxylin-eosin-stained sections of anterior prostate from genetically engineered mouse models. (*E*) Normal tissue, with characteristic papillary tufting (arrowheads). (*F*) High-grade PIN. (*G*) Prostate carcinoma. (*H*) Prostate carcinoma with an invasive phenotype. We thank Dr. Robert Cardiff and Alexander Borowsky (School of Medicine, University of California at Davis) for providing images of human prostate specimens.

(Grignon 2004). Perhaps the most significant histological variant is neuroendocrine prostate cancer, which is generally classified as either small cell carcinoma or a carcinoid tumor, and represents <2% of prostate cancer cases (Grignon 2004). However, focal regions of neuroendocrine differentiation are more commonly observed in prostate adenocarcinoma, particularly following recurrence after prostatectomy and androgen deprivation therapy (Yuan et al. 2007; Komiya et al. 2009), and expression of the neuroendocrine marker chromogranin A is associated with the development of castration-resistant tumors and shortened time to disease recurrence (Kokubo et al. 2005; Berruti et al. 2007). This prevalence of neuroendocrine differentiation after recurrence may be due to the lack of AR expression by neuroendocrine cells, which are inherently castration-resistant.

While evidence of major subtypes of prostate cancer is lacking at the histopathological level, recent genomic analyses have provided increasing evidence for molecularly defined subtypes (Tomlins et al. 2008b; Palanisamy et al. 2010; Taylor et al. 2010). In particular, expression profiling analyses of prostate cancer specimens have not strictly defined molecular signatures associated with distinct cancer subtypes that specifically correlate with disease outcome (Singh et al. 2002; Lapointe et al. 2004; Tomlins et al. 2007b). However, oncogenomic pathway analyses that integrate analyses of gene expression, copy number alterations, and exon resequencing may provide a unified approach for distinguishing prostate cancer subtypes and stratifying patient outcome (Taylor et al. 2010). Furthermore, the existence of molecular subtypes has been supported by analyses of chromosomal rearrangements associated with prostate cancer; for example, prostate cancers containing the *TMPRSS2-ERG* translocation may be distinct from those that up-regulate *SPINK1,* which encodes a secreted trypsin inhibitor (Mehra et al. 2007a; Tomlins et al. 2008b). Ongoing studies will undoubtedly assess whether these subtypes correlate with disease outcome or treatment response.

Metastasis

As noted above, although common sites of secondary metastasis for prostate cancer are lung, liver, and pleura, if prostate cancer metastasizes, it invariably goes to bone, where it forms characteristic osteoblastic lesions (Bubendorf et al. 2000; Logothetis and Lin 2005). Given the clinical importance of metastasis for patient outcome, the ability of prostate tumor cells to disseminate into the bone marrow and peripheral blood has been investigated in detail. Notably, a recent study showed that bona fide circulating tumor cells could be detected in the bone marrow of a significant proportion of patients with localized disease, suggesting that disseminated tumor cells have not attained full metastatic capability (Holcomb et al. 2008). Consistent with this interpretation, circulating tumor cells from patients with metastatic disease show multiple chromosomal rearrangements typical of advanced prostate cancer, consistent with genomic instability acquired during cancer progression (Holcomb et al. 2008; Attard et al. 2009c; Leversha et al. 2009). However, the relationship of disseminated tumor cells to the formation of metastases remains unresolved, and the molecular factors that promote metastases of prostate cancer to bone are poorly defined.

Mouse models of prostate cancer

Xenograft models

Traditionally, in vivo studies of prostate cancer have extensively used xenograft models of human prostate cancer, using cell lines or prostate tumors implanted into immunodeficient mice, either orthotopically into the prostate or transplanted onto the flank. For instance, xenografts derived from LNCaP cells have been used to generate genetically-related lines that vary in their androgen responsivity and metastatic potential (Thalmann et al. 2000). Similarly, several xenografts have been developed by transplantation of human prostate tumors, including the LuCaP and LAPC series, which display a spectrum of prostate cancer phenotypes (Ellis et al. 1996; Craft et al. 1999b). Several of these human tissue xenografts have also given rise to prostate cancer cell lines, such as the VCaP line, which was derived from a bone metastasis (Korenchuk et al. 2001).

Analyses of xenografts have yielded a vast amount of information about molecular mechanisms of prostate cancer, and have been useful for chemotherapeutic approaches. However, xenograft models are limited by a heterologous microenviroment (since human cells/tissues are grafted in mice), an inability to analyze stromal components (unless orthotopic grafting is employed), the lack of endogenous immune response (since the host mice are immunodeficient), and the lack of diversity of available established cell lines. In particular, the limited number of available prostate cancer cell lines is likely related to inherent difficulties in culturing luminal epithelial cells (Peehl 2005) (existing prostate cell lines have been reviewed in detail) (Sobel and Sadar 2005a,b). Of particular concern is that existing cell lines may have uncertain origins, as has been demonstrated for at least one "prostate" cell line that was actually derived from a bladder carcinoma line (van Bokhoven et al. 2001). Additionally, cell lines may have anomalous molecular properties (e.g., loss of AR expression and lack of *TMPRSS2-ERG* fusions) when compared with most human prostate tumors (Sobel and Sadar 2005a,b), which may limit their applicability. Nonetheless, xenograft systems remain popular for studies of chemotherapeutics, primarily due to their ease of use. Moreover, since they are of human origin, xenografts may be more likely to recapitulate molecular events involved in human prostate tumorigenesis than other experimental models.

Tissue reconstitution models

The tissue recombination and renal grafting methods originally developed to study epithelial–mesenchymal interactions during prostate organogenesis can be extended for investigation of prostate tumorigenesis in vivo. In particular, immortalized human and mouse prostate

epithelial cell lines are available that can reconstitute benign prostate tissue with relatively normal histology following recombination with rodent embryonic urogenital mesenchyme and grafting into immunodeficient recipients (Hayward et al. 2001; Gao et al. 2004a; Jiang et al. 2010). Using such cell lines, gene expression can be directly manipulated in culture by overexpression or knockdown methods, followed by analysis of potential tumor phenotypes in vivo. Similarly, stromal components can also be investigated in tissue recombinants using immortalized urogenital mesenchyme cell lines to facilitate genetic manipulation (Shaw et al. 2006), or using carcinoma-associated fibroblasts (Olumi et al. 1999). Furthermore, this overall approach can been greatly extended by efficient lentiviral infection of dissociated prostate epithelial cells, followed by tissue recombination, allowing for overexpression and knockdown approaches for analysis of gene function (Xin et al. 2003, 2006; Zong et al. 2009). Finally, grafting methods can be used to evaluate the relative contribution of epithelial and stromal prostatic components for cancer progression (Kim et al. 2002a; Jeong et al. 2008), as well as to study cancer phenotypes in the prostate glands of mouse strains that display embryonic lethality, even prior to prostate formation (Wang et al. 2000). Thus, tissue reconstitution methods represent powerful approaches to studying cancer mechanisms in both mouse and human prostates.

Genetically engineered models

The use of genetically engineered transgenic and knockout mice to produce autochthonous models of prostate cancer has represented a major avenue for prostate cancer investigations (Table 1). Most first-generation prostate cancer models used transgenes that overexpress potent viral oncogenes, resulting in highly aggressive disease that can often lead to metastatic cancer (Winter et al. 2003; Kasper 2005). Among these models are the well-studied TRAMP (transgenic adenocarcinoma of the prostate) mouse, which carries a minimal probasin promoter driving both SV40 large T and small t antigen (Greenberg et al. 1995), and the LADY models that use a larger probasin promoter and express large T antigen only (Masumori et al. 2001). However, transgenic mice that overexpress SV40 large T antigen typically have short latency, and develop cancer with features of neuroendocrine differentiation (Kaplan-Lefko et al. 2003; Shappell et al. 2004). Nonetheless, these first-generation models have provided numerous important insights into prostate cancer mechanisms.

A second generation of prostate cancer models has used loss-of-function mutations in candidate genes implicated in the genesis of human prostate cancer (Table 1). Several popular models have employed null mutations in genes of interest, including *Nkx3.1* and *Pten* (*phosphatase and tensin homolog deleted from chromosome 10*) (discussed below); for example, *Nkx3.1; Pten* double mutants show accelerated formation of high-grade PIN and invasive cancer (Kim et al. 2002d; Abate-Shen et al. 2003). Other commonly used models have employed conditional deletion mediated by the *Pb-Cre4* transgene, which uses a modified probasin promoter (ARR_2PB) to drive Cre expression in the prostate epithelium (Wu et al. 2001), although a potential concern is that this Cre allele also drives recombination in the stroma (X Wang and MM Shen, unpubl.). The *Pb-Cre4* driver has been used by many laboratories for the conditional deletion of *Pten* as well as other genes of interest (Wang et al. 2003; Z Chen et al. 2005; Bruxvoort et al. 2007). Another popular Cre driver is the *Nkx3.1-Cre* knock-in allele, which expresses Cre recombinase specifically in the prostate epithelium, but also in several other tissues during embryogenesis (Stanfel et al. 2006; Lin et al. 2007; Thomsen et al. 2008; Zhang et al. 2008).

Despite much recent progress, current genetically engineered models have several important limitations. First, constitutive conditional gene deletion systems generally result in deletion from early stages of prostate organogenesis, and cannot be initiated in the adult, or in a stochastic manner, as is the case with somatic mutations in human cancer. Thus, the development of an inducible gene targeting system that can be induced in adult prostate epithelium would allow the investigation of gene function in situations where normal prostate organogenesis would not proceed in the absence of the gene of interest. Recent publications have described the generation of tamoxifen-inducible Cre drivers that are likely to be suitable for such approaches (Luchman et al. 2008; Ratnacaram et al. 2008; Birbach et al. 2009; Z Wang et al. 2009). Second, the use of a reversible transgene expression system, such as those driven by a tetracycline-regulated promoter, would allow the modeling of targeted therapeutic interventions on cancer growth, as well as potential acquisition of drug resistance and treatment failure. Such tetracycline-regulated models have been used successfully to investigate oncogene addiction in melanoma and mammary cancer models (Chin et al. 1999; Moody et al. 2002), but have not yet been employed in the prostate. Third, existing Cre drivers to investigate stromal function in prostate carcinogenesis are limited and nonspecific (Jackson et al. 2008), with the best available driver corresponding to the *FSP1-Cre* transgene (Bhowmick et al. 2004). Fourth, all current prostate cancer models use androgen-dependent promoters to drive the cancer phenotype, either directly or indirectly, and thus are poorly suited to investigate the effects of modulating androgen levels, since androgen deprivation will simultaneously downregulate transgene expression. Finally, at present, there is no autochthonous model that reliably displays bone metastasis, which represents a major limitation in the study of advanced prostate cancer.

Nonetheless, despite their limitations, analyses of genetically engineered mouse models of prostate cancer have significantly advanced our understanding of the molecular pathways of prostate cancer initiation, progression, and castration resistance. Additionally, investigations of genetically engineered mice have led to the identification of biomarkers that can predict disease recurrence, and have provided valuable preclinical resources for investigations of novel therapies and analyses

Table 1. *Representative genetically engineered mouse models of prostate cancer*[a]

Type	Name	Description	Reference
Gain-of-function transgenic models	*TRAMP (rPB-SV40)*	SV40 large tumor antigen (Tag) driven by a minimal rat probasin promoter (rPB). Phenotype: PIN, adenocarcinoma, neuroendocrine differentiation, and metastases; castration-resistant prostate cancer.	Greenberg et al. 1995
	Lady (LPB-Tag)	SV40 large tumor antigen driven by large probasin promoter. Phenotype: PIN, adenocarcinoma, neuroendocrine differentiation, and metastases.	Masumori et al. 2001
	TgAPT$_{121}$ (ARR2PB-APT$_{121}$)	Truncated SV40 T antigen (without small t antigen) driven by minimal probasin promoter with androgen-regulated sites (ARR2PB). Phenotype: PIN and adenocarcinoma.	Hill et al. 2005
	Hi-Myc/Low-Myc (ARR2PB-c-Myc)	c-Myc driven by ARR2PB promoter; Hi-Myc and low-Myc differ in latency. Phenotype: PIN and adenocarcinoma.	Ellwood-Yen et al. 2003
	MPAKT (rPB-myr-HA-Akt1)	Myristoylated Akt1driven by the rPB promoter. Phenotype: PIN.	Majumder et al. 2003
	iBraf (Tet-BRAF^{V600E}; Tyr-rtTA; Ink4a/Arf$^{-/-}$)*	A human mutant B-RAF (V600E) driven by the Tet promoter and crossed with mice having a tet-regulatable tyrosinase promoter. Phenotype: PIN and adenocarcinoma.	Jeong et al. 2008
Loss-of-function models	*Nkx3.1$^{+/-}$* and *Nkx3.1 $^{-/-}$* and *Nkx3.1$^{flox/flox}$*	Germline deletion of *Nkx3.1* (or conditional deletion of *Nkx3.1* in the germline). Phenotype: PIN.	Bhatia-Gaur et al. 1999; Abdulkadir et al. 2002; Kim et al. 2002b
	Pten$^{+/-}$	Germline deletion of *Pten*. Phenotype: PIN and high-grade PIN; castration-resistant prostate cancer. Phenotypes not restricted to prostate.	Di Cristofano et al. 1998b; Podsypanina et al. 1999
	Nkx3.1$^{+/-}$; Pten$^{+/-}$	Compound germline mutant mice; Phenotype: PIN, adenocarcinoma, metastases to lymph nodes; castration-resistant prostate cancer. Phenotypes not restricted to prostate.	Kim et al. 2002c; Abate-Shen et al. 2003
	Pten$^{+/-}$; p27$^{-/-}$ and *Nkx3.1$^{+/-}$; Pten$^{+/-}$; p27$^{+/-}$*	Compound germline mutant mice. Phenotype: PIN, adenocarcinoma. Phenotypes not restricted to prostate.	Di Cristofano et al. 2001; Gao et al. 2004b
	TMPRS-Erg; Pten$^{+/-}$	Germline loss of function of *Pten* combined with gain of function of the TMPRS-Erg transgene. Phenotype: PIN, adenocarcinoma.	Carver et al. 2009; King et al. 2009
Conditional loss-of-function models	*PB-Cre; Pten$^{flox/flox}$*	Conditional deletion of *Pten* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma; castration-resistant prostate cancer.	Trotman et al. 2003; Wang et al. 2003
	PSA-Cre-ER^{t2}; Pten$^{flox/flox}$	Conditional deletion of *Pten* in the prostate driven by a PSA promoter driving an inducible Cre-ER^{T2} recombinase. Phenotype: PIN, adenocarcinoma.	Ratnacaram et al. 2008
	PB-Cre; Pten$^{flox/flox}$; p53$^{flox/flox}$	Conditional deletion of *Pten* and *p53* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma.	Z Chen et al. 2005

(continued)

Table 1. *continued*

Type	Name	Description	Reference
	PB-Cre; APC	Conditional deletion of *APC* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma; castration-resistant prostate cancer.	Bruxvoort et al. 2007
	PB-Cre; Pten$^{flox/flox}$, Z-Myc	Conditional activation of Myc plus conditional deletion of Pten in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma.	Kim et al. 2009
	PB-Cre; p53$^{flox/flox}$; Rb$^{/flox}$	Conditional deletion of *p53* and *Rb* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma, neuroendocrine differentiation.	Zhou et al. 2006
	AhCre; LKB$^{flox/flox}$	Conditional deletion of LKB by activation of a p450 CYP1A1-driven Cre recombinase transgene (AhCre). Phenotype: PIN.	Liao et al. 2007
AR signaling	*PB-AR*	Mouse AR transgene driven by a probasin promoter. Phenotype: PIN.	Stanbrough et al. 2001
	rPB-AR-T877A and *rPB-AR-E231G*	Mouse AR transgene with a mutation in either T877A or E231G driven by a minimal probasin promoter. Phenotype (of E231G): PIN, adenocarcinoma.	Han et al. 2005
	Pes-ARKO	Conditional deletion of a floxed AR allele driven by a probasin promoter driving Cre recombinase. Phenotype: hyperproliferation.	Wu et al. 2007
FGF signaling	*PB-FGF7 (PKS)* *PB-FGFR2iib (KDNR)*	FGF7(PKS) or a dominant-negative FGFR2iib transgene driven by minimal probasin promoter. Phenotype: PIN; KDNR develops neuroendocrine differentiation.	BA Foster et al. 2002
	ARR2PB-FGF8	FGF8 transgene driven by ARR2PB promoter. Phenotype: PIN.	Song et al. 2002
	PB-FGF-R1(K656E) *PB-FGFR1; KDNR*	Enforced expression of a mutant (activated) form of FGF receptor R1 by a minimal probasin promoter, alone or with a dominant-negative FGFR2. Phenotype: PIN.	Jin et al. 2003; Wang et al. 2004
	iFGF-R1	Chemically inducible FGFR1 in prostate. Phenotype: PIN, adenocarcinoma.	Freeman et al. 2003; Acevedo et al. 2007

[a]Selected models represent the range of phenotypes, transforming events, and allelic alterations that are available for studying prostate cancer in GEM mice.

of chemopreventive agents. Examples of each of these applications are described below.

Processes that promote prostate carcinogenesis

The single most significant risk factor for prostate cancer is advanced age. While men who are younger than 40 have a one in 10,000 chance of developing prostate cancer, this risk increases to one in seven by the age of 60 (American Cancer Society 2009). However, prostate cancer is not simply a by-product of aging, since the incidence varies considerably among different populations. More likely, the relationship of prostate cancer to advanced age likely reflects the interplay of environmental, physiological, and molecular influences with normal consequences of aging that presumably exacerbate the effects of these influences. Moreover, while the precise molecular consequences of aging as they pertain to prostate cancer have not been elucidated, various studies have described gene expression changes associated with aging, particularly in the prostatic stroma, including genes involved in inflammation, oxidative stress, and cellular senescence (Begley et al. 2005; Bavik et al. 2006; Bethel et al. 2009).

Below, we discuss some of the major processes that have been implicated in prostate carcinogenesis (Fig. 1). Perhaps not surprisingly, these are interrelated and involve key regulatory molecules that have been associated with cancer initiation and progression, which will be discussed in the next section.

Inflammation

Various lines of epidemiological, pathological, and molecular evidence have supported the idea that chronic inflammation is causally linked to prostate carcinogenesis (Haverkamp et al. 2008; Klein and Silverman 2008; Bardia et al. 2009). For example, expression of certain chemokines is a predictor of biochemical disease recurrence in human prostate cancer (Blum et al. 2008). Moreover, administration of the potent heterocyclic amine PhIP (2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine), results in chronic inflammation and promotes prostatic hyperplasia and PIN in rodents (Borowsky et al. 2006; Elkahwaji et al. 2007; Nakai et al. 2007; Elkahwaji et al. 2009; Khalili et al. 2010). One of the most well-studied genes involved in protection against carcinogens such as PhIP is *GSTP1*, which encodes a member of the glutathione S-transferase family that is responsible for detoxification of reactive species, and is epigenetically silenced in a majority of prostate cancers by DNA methylation (Nakayama et al. 2004). Most notably, regions of focal atrophic prostate epithelium can often be identified in aging men, frequently in association with an inflammatory response. Such regions usually display increased epithelial proliferation, and have been termed "proliferative inflammatory atrophy" (PIA) (De Marzo et al. 1999). Regions of PIA are also often located in proximity with PIN and adenocarcinoma, and thus PIA has also been proposed to represent a precursor lesion for prostate cancer (De Marzo et al. 1999, 2003).

These observations have raised interest in the potential causes of prostate inflammation, which may include hormonal perturbations such as altered androgen and estrogen levels, or infection by bacterial or viral agents, physical trauma, or dietary factors (De Marzo et al. 2007b). Indeed, the susceptibility of the prostate gland to infection is known from the incidence of chronic bacterial prostatitis, and a potential role for bacterial infection in prostate carcinogenesis has been suggested by the identification of multiple bacterial species in most prostatectomy samples examined (Sfanos et al. 2008). Interestingly, a recent study has shown that induced bacterial prostatitis in mice can result in histological changes reminiscent of human PIA and down-regulation of expression of the Nkx3.1 homeoprotein (Khalili et al. 2010), while the levels of NKX3.1 expression in human prostate cancer cell lines have been shown to be regulated by inflammatory cytokines (Markowski et al. 2008).

Support for a role for viral infection as contributor to chronic inflammation of the prostate has been provided by studies of rare hereditary prostate cancer (HPC) that have identified several loci associated with increased familial prostate cancer risk (Schaid 2004). Of these loci, *HPC1* has been shown to correspond to *RNASEL*, which encodes 2′-5′-oligoadenylate (2-5A)-dependent RNase L, an endoribonuclease for ssRNA that is a component of the interferon response to viral infection (Carpten et al. 2002). Further studies have shown that the R462Q variant of *RNASEL* confers decreased protein activity and is associated with increased risk of sporadic prostate cancer (Casey et al. 2002; Xiang et al. 2003), although the relevance of this allele as well as other *RNASEL* variants for sporadic prostate cancer has been disputed (Wiklund et al. 2004; Li and Tai 2006). Notably, a significant percentage of prostate tumors from patients carrying the variant *RNASEL* allele have been reported to contain a novel gammaretrovirus, termed xenotropic murine leukemia virus-related virus (XMRV) (Urisman et al. 2006; Dong et al. 2007), which is expressed in ~20% of prostate cancer samples, but is not correlated with the presence of the *RNASEL* R462Q variant (Schlaberg et al. 2009). At present, there is considerable interest in the possibility that XMRV infection may play a role in prostate cancer initiation through stimulation of an inflammatory response, but further studies are required to replicate these initial findings and evaluate a possible causal link (Silverman et al. 2010).

Oxidative stress and DNA damage

Several lines of evidence have suggested that one of the major aging-associated influences on prostate carcinogenesis is oxidative stress and its cumulative impact on DNA damage (DeWeese et al. 2001; Khandrika et al. 2009; Minelli et al. 2009). Oxidative stress results from the imbalance of reactive oxygen species (ROS) and detoxifying enzymes that control cellular levels of ROS, which leads to cumulative damage to lipids, proteins, and DNA. The prostate appears to be exceptionally vulnerable to oxidative stress, perhaps as a consequence of inflammation,

hormonal deregulation, diet, and/or epigenetic modifications such as silencing of *GSTP1*. Evidence linking oxidative stress and prostate cancer initiation include correlative studies showing that major antioxidant enzymes are reduced in human PIN and prostate cancer, together with a coincidental increase in the oxidized DNA adduct 8-oxy-7,8,dihydro-2′-deoxyguanosine (8-oxy-dG) (Bostwick et al. 2000). Furthermore, APE/Ref1, a multifunctional enzyme involved in redox control of key enzymes and base excision repair, is up-regulated in prostate cancer, while polymorphisms in the *APE* gene are associated with increased prostate cancer risk (Kelley et al. 2001; L Chen et al. 2006). Similarly, perturbations in oxidative stress response pathways have been observed in genetically engineered mouse models of prostate cancer coincident with cancer progression (Ouyang et al. 2005; Frohlich et al. 2008). Interestingly, loss of function of the *Nkx3.1* homeobox gene in the mouse prostate leads to deregulated expression of oxidative damage response genes and increased levels of 8-oxy-dG, correlated with the onset of PIN (Ouyang et al. 2005), while gain of function of the *NKX3.1* homeobox gene has also been shown to protect against DNA damage in prostate cancer cell lines (Bowen and Gelmann 2010). Since *NKX3.1* is frequently down-regulated in early stages of prostate cancer, its inactivation may contribute to the observed vulnerability of the prostate to oxidative stress as well as to DNA damage associated with cancer initiation.

Telomere shortening

Another event that has been implicated in prostate cancer initiation is the shortening of telomeres, which is generally associated with DNA damage and may lead to chromosomal instability (Meeker et al. 2002, 2004; Vukovic et al. 2003). Telomere length has been correlated with disease outcome (Fordyce et al. 2005; Joshua et al. 2007), while prostate carcinomas as well as many high-grade PINs display increased telomerase activity, which is not observed in benign prostate tissue (Sommerfeld et al. 1996; Koeneman et al. 1998). These observations suggest that telomere length is actively modulated during prostate cancer progression, but the mechanistic relationships between telomere shortening and cancer initiation, or to induction of cellular senescence (see below), are presently unclear. Nonetheless, various strategies to regulate telomere length are being investigated as potential therapeutic agents (Asai et al. 2003; Chen et al. 2003).

Senescence

Cellular senescence corresponds to a form of cell cycle arrest in which cells remain fully viable, but are non-proliferative despite exposure to mitogenic signals (Courtois-Cox et al. 2008; d'Adda di Fagagna 2008; Evan and d'Adda di Fagagna 2009). Much recent work has identified cellular senescence as a potent mechanism of tumor suppression that prevents manifestation of the malignant phenotype after oncogenic insults. In particular, activated oncogenes are believed to induce senescence through a variety of molecular mechanisms, including replicative stress or formation of ROS, or as a response to DNA damage. Thus, oncogene-induced senescence may play a central role in preventing the progression of preneoplastic lesions to the fully malignant state.

In the prostate, cellular senescence has been shown to occur during aging-related prostate enlargement, and has been implicated as a tumor suppressor mechanism for prostate carcinogenesis. Thus, SA-β-Gal, a commonly used biomarker of senescence, is frequently detected in BPH in the human prostate (Choi et al. 2000; Castro et al. 2003). Moreover, other markers of senescence, including $p14^{arf}$ and $p16^{ink4a}$, are increased with aging and particularly in nonmalignant cancers, suggesting these may represent markers that distinguish indolent from more aggressive forms of the disease (Zhang et al. 2006). In addition to senescence-related changes observed in epithelial cells, senescent primary prostatic fibroblasts display gene expression signatures associated with oxidative damage and DNA damage, which may in turn influence the invasive behavior of epithelial cells (Bavik et al. 2006). Notably, gene expression changes affecting oxidative damage and DNA damage responses are also observed in prostatic stroma from aged, tumor-prone rats (Bethel et al. 2009), as well as in reactive stroma from human prostate tumors (Dakhova et al. 2009).

Studies in genetically engineered mice have provided mechanistic insights into the role of senescence for prostate tumorigenesis. In particular, complete conditional inactivation of the *Pten* tumor suppressor gene results in PIN lesions that display a senescence phenotype that can be overcome by inactivation of *p53* (Z Chen et al. 2005), but is enhanced in combination with inactivation of the *Skp2* E3-ubiquitin ligase (Lin et al. 2010). Based on these findings in mouse models, one possible interpretation for the temporal difference between the occurrence of latent prostate cancer and the appearance of clinical prostate cancer is that cellular senescence may be involved in suppressing progression to aggressive disease, while additional oncogenic events may be required to bypass the senescence mechanism to promote disease progression.

Genomic alterations

Extensive genomic analyses of prostate cancer have identified copy number alterations and chromosomal rearrangements associated with prostate carcinogenesis. In particular, a number of important somatic alterations have been identified by comparative genomic hybridization (CGH) as gains or losses of chromosomal regions, including gains at 8q and losses at 3p, 8p, 10q, 13q, and 17p (Dong 2001; Lapointe et al. 2007; Taylor et al. 2010). Importantly, several of these genetic alterations have also been identified in PIN as well as PIA lesions, which has supported the precursor relationship of these lesions to prostate cancer and has emphasized their relevance for promoting cancer progression. Finally, several key regulatory genes have been mapped to within these chromosomal regions undergoing copy number alterations, including *NKX3.1* at 8p21, *PTEN* at 10q23, and MYC at 8q24. In contrast, however, targeted resequencing studies have suggested that somatic point mutations may be relatively

infrequent in prostate cancer, with tumor suppressor genes such as *TP53* undergoing alterations of copy number instead (Taylor et al. 2010).

Genetic factors

Extensive efforts have been made to identify genetic susceptibility loci for prostate cancer, both through analyses of hereditary factors associated with familial risk of early-onset disease, and more recently through genome-wide association studies. In particular, prostate cancer susceptibility loci associated with HPC have been mapped to 1q24-25 (HPC1), 17p11 (HPC2), and Xq27-28 (HPCX) (Xu et al. 1998, 2001a,b), which correspond to *RNASEL* (HPC1), which was discussed above, and *ELAC* (HPC2), a gene of uncertain function. Additionally, genome-wide association studies have identified numerous single-nucleotide polymorphisms (SNPs) that are associated with cancer risk (Thomas et al. 2008; Eeles et al. 2009; Gudmundsson et al. 2009; Kader et al. 2009). In particular, a major locus identified in these genome-wide association studies is identified by multiple sequence polymorphisms at 8q24, proximal to *MYC*, and is discussed below. Disappointingly, however, many of the other loci identified in genome-wide association studies have not been easily replicated in other population-based studies, including analyses of groups with high-risk for prostate cancer, such as African-Americans (Hooker et al. 2010). Finally, studies of individual genetic loci have identified rare sequence polymorphisms associated with increased cancer risk, as has been shown for the *C154T* polymorphism at the *NKX3.1* locus (Gelmann et al. 2002).

Epigenetic alterations

Epigenetic perturbations are also believed to represent important contributing factors in prostate carcinogenesis, and may provide useful biomarkers for disease progression (Li et al. 2005; Nelson et al. 2007, 2009). For example, DNA methylation has been implicated in silencing genes involved in signal transduction, hormonal response, cell cycle control, and oxidative damage response, such as *GSTP1*. Furthermore, prostate tumors display global changes in chromatin modification coincident with cancer progression (Kondo et al. 2008; Ke et al. 2009) that presumably result in significant perturbations in the gene expression program of tumor cells. One key modification associated with prostate carcinogenesis is trimethylation of lysine residue 27 of histone H3 (H3K27-me3), which is mediated by the histone methyltransferase enzyme Ezh2, a key oncogenic driver of advanced disease and metastasis (Varambally et al. 2002). Since the H3K27-me3 mark is associated with transcriptional repression, increased levels in prostate cancer are associated with repression of tumor suppressor genes such as DAB2IP, a member of the Ras GTPase family (H Chen et al. 2005). Global changes in histone modifications are also associated with cellular senescence, through the development of senescence-associated foci (SAHF), which include epigenetic marks of chromatin silencing (Funayama and Ishikawa 2007). In the future, global analyses of histone modifications by next-generation sequencing approaches may provide broad insights on the cumulative influences of these modifications for prostate carcinogenesis.

Molecular mechanisms of prostate cancer initiation and progression

Below, we discuss several molecular events that are believed to occur in a large percentage of prostate carcinomas, focusing on their relationships to key processes discussed in the preceding section (Fig. 1). Although each event has been associated with a possible role in cancer initiation or progression, it is unknown whether there is a temporal sequence associated with these events, or whether there is a causal relationship between them.

NKX3.1 down-regulation

Down-regulation of the *NKX3.1* homeobox gene represents a frequent and critical event in prostate cancer initiation, and is likely to involve multiple mechanisms (Abate-Shen et al. 2008). *NKX3.1* is localized within a 150-Mb minimal deleted region of chromosome 8p21.2 that displays loss-of-heterozygosity (LOH) in up to 85% of high-grade PIN lesions and adenocarcinomas (Emmert-Buck et al. 1995; Vocke et al. 1996; Haggman et al. 1997; Swalwell et al. 2002; Bethel et al. 2006). However, although LOH of 8p21 progressively increases in frequency with cancer grade, the remaining allele of *NKX3.1* remains unmutated (Vocke et al. 1996; Voeller et al. 1997; Ornstein et al. 2001; Bethel et al. 2006). In addition, whether or not 8p21 LOH has occurred, there is substantial evidence that *NKX3.1* undergoes epigenetic down-regulation, perhaps through promoter methylation (Asatiani et al. 2005). Although earlier studies had suggested that NKX3.1 expression is completely lost in advanced cancers (Bowen et al. 2000), recent analyses using a highly sensitive antibody indicate that low levels of NKX3.1 expression can be demonstrated in nearly all prostate cancers and metastases examined (Gurel et al. 2010). Thus, there appears to be a selection for reduction, but not loss, of NKX3.1 expression throughout prostate cancer progression.

These findings are highly suggestive, since *Nkx3.1* has been shown to be a critical regulator of prostate epithelial differentiation and stem cell function in mouse models. During development, *Nkx3.1* is expressed in all epithelial cells of the nascent prostate buds from the urogenital sinus, and represents the earliest known marker for the prostate epithelium (Bhatia-Gaur et al. 1999). In the absence of *Nkx3.1,* there is a significant decrease in prostatic ductal branching, as well as in production of secretory proteins (Bhatia-Gaur et al. 1999; Schneider et al. 2000; Tanaka et al. 2000). Notably, young adult *Nkx3.1* heterozygous and homozygous mutants frequently display prostate epithelial hyperplasia and dysplasia, and often develop intraductal neoplasia (PIN) by 1 year of age (Bhatia-Gaur et al. 1999; Schneider et al. 2000; Tanaka et al. 2000; Abdulkadir et al. 2002; Kim et al. 2002a). These findings are consistent with the tumor suppressor activity of *NKX3.1* in cell culture and xenograft assays (Kim et al.

2002a; Lei et al. 2006). Finally, recent work has shown that *Nkx3.1* expression in the androgen-deprived prostate marks a rare population of prostate epithelial stem cells that is a cell of origin for prostate cancer in mouse models (Z Wang et al. 2009).

Analyses of *Nkx3.1* function in human tumor cells and genetically engineered mice have provided insights into its potential roles in cancer initiation. In particular, *Nkx3.1* inactivation in mice results in a defective response to oxidative damage, while its expression in human prostate cancer cell lines protects against DNA damage and is regulated by inflammation (Ouyang et al. 2005; Markowski et al. 2008; Bowen and Gelmann 2010). A causal role for *Nkx3.1* in these processes has been suggested by analyses of genes that are dysregulated following perturbation of *Nkx3.1* expression in mouse models or human cell lines (Magee et al. 2003; Ouyang et al. 2005; Muhlbradt et al. 2009; Song et al. 2009). These and other findings have led to a model in which *NKX3.1* represents a haploinsufficient tumor suppressor gene that acts as a "gatekeeper" gene for prostate cancer initiation (Kim et al. 2002a; Gelmann 2003; Magee et al. 2003).

Myc up-regulation

It has long been known that the 8q24 chromosomal region encompassing the *MYC* oncogene is somatically amplified in a subset of advanced prostate tumors (Jenkins et al. 1997; Sato et al. 1999). However, recent studies have suggested a role for *MYC* overexpression in cancer initiation, as nuclear MYC protein is up-regulated in many PIN lesions and the majority of carcinomas in the absence of gene amplification (Gurel et al. 2008). These findings may be consistent with the identification of a major susceptibility locus at 8q24 in several large-scale genome-wide association studies of prostate cancer as well as other epithelial cancers (Amundadottir et al. 2006; Freedman et al. 2006; Gudmundsson et al. 2007, 2009; Haiman et al. 2007; Yeager et al. 2007, 2009; Al Olama et al. 2009). Multiple SNPs associated with prostate cancer risk alleles are clustered within three independent regions of a gene-poor genomic locus spanning ~1.2 Mb between *FAM84B* and *MYC,* with *MYC* located ~250 kb away from the closest SNP marker. Detailed analyses have not yet revealed any correlation between risk alleles and *MYC* RNA expression levels in prostate tumor samples, or the presence of any non-protein-coding genes such as microRNAs (miRNAs) (Pomerantz et al. 2009). Nonetheless, long-range regulatory elements for *MYC* have been identified recently in this region, raising the possibility that the risk alleles may alter the regulation of *MYC* expression (Jia et al. 2009; Sotelo et al. 2010). Interestingly, another recent study has found that the X-linked gene *FOXP3* encodes a winged helix transcription factor that represses *MYC* expression (although apparently not through distant enhancer-binding sites), and itself is mutated in prostate cancer (L Wang et al. 2009).

At the functional level, transgenic mice overexpressing human *MYC* display rapid formation of PIN followed by progression to invasive adenocarcinoma with rare metastases (Ellwood-Yen et al. 2003), while forced expression of *MYC* is sufficient to immortalize nontumorigenenic human prostate epithelial cells (Gil et al. 2005). Interestingly, bioinformatic analyses identified an expression signature characterized by down-regulation of *Nkx3.1* and up-regulation of *Pim1,* which is known to collaborate with *Myc* in lymphomas (Ellwood-Yen et al. 2003). Consistent with these data, lentiviral coexpression of human *MYC* with mouse *Pim1* in tissue recombinants results in cooperative formation of carcinomas with neuroendocrine differentiation (Wang et al. 2010).

TMPRSS2-ERG translocations

Important recent studies have identified chromosomal rearrangements that activate members of the *ETS* family of transcription factors (ERG, ETV1, and ETV4) in the majority of prostate carcinomas (Tomlins et al. 2005, 2007a; Iljin et al. 2006; Mehra et al. 2007b; Mosquera et al. 2007; Hu et al. 2008; Rouzier et al. 2008; Saramaki et al. 2008). The most common of these rearrangements creates a *TMPRSS2-ERG* fusion gene, resulting in expression of N-terminally truncated ERG protein under the control of the androgen-responsive promoter of *TMPRSS2* (Tomlins et al. 2005; Iljin et al. 2006; Perner et al. 2006; J Wang et al. 2006; Clark et al. 2007). As *TMPRSS2* and *ERG* are located ~3 Mb apart on chromosome 21q, this rearrangement occurs through either an interstitial deletion, which is more common, or an unbalanced interchromosomal translocation (Iljin et al. 2006; Perner et al. 2006). The frequency of these *TMPRSS2-ERG* fusions is ~15% in high-grade PIN lesions, and ~50% in localized prostate cancer (Clark et al. 2008; Mosquera et al. 2008; Albadine et al. 2009), suggesting that this rearrangement either occurs after cancer initiation, or alternatively corresponds to an early event that predisposes to clinical progression. Interestingly, formation of these chromosomal rearrangements may be an indirect consequence of AR function, as studies in androgen-responsive LNCaP cells have shown that AR binding induces chromosomal proximity between the *TMPRSS2* and *ERG* loci that can lead to formation of *TMPRSS2-ERG* fusions following DNA damage (Lin et al. 2009; Mani et al. 2009). In addition, androgen signaling can recruit topoisomerase II to AR-binding sites, leading to induction of double-stranded breaks even in the absence of genotoxic stress (Haffner et al. 2010).

Despite the prevalence of these genomic rearrangements, the functional significance of the *TMPRSS2-ERG* fusion and other *ETS* rearrangements in prostate cancer is still not fully resolved. Recent whole-genome chromatin immunoprecipitation analyses have shown that ERG can bind to AR downsteam target genes and disrupts AR signaling in prostate cancer cells through epigenetic silencing, consistent with a role in inhibiting prostate epithelial differentiation (Yu et al. 2010). Furthermore, analyses of *ETS* gene activation in cell culture assays as well as transgenic mice have suggested that *ETS* activation promotes EMT and tumor-invasive properties (Tomlins et al. 2007a, 2008a; Klezovitch et al. 2008; J Wang et al. 2008), although the effects are relatively moderate. In transgenic mice, expression of truncated human *ERG* transgenes results in a minimal or weak PIN phenotype

(Tomlins et al. 2007a, 2008a; Klezovitch et al. 2008). However, expression of truncated *ERG* synergizes with loss of *Pten* to result in high-grade PIN and carcinoma in mice (Carver et al. 2009; King et al. 2009). In addition, recent findings suggest that *TMPRSS2-ERG*-positive tumors are also associated with the deletion of a small genomic region on 3p14, suggestive of another cooperative interaction in tumorigenesis (Taylor et al. 2010). Taken together, these findings suggest that *ETS* rearrangements are selected primarily for their ability to disrupt differentiation programs and/or to promote prostate cancer progression through cooperative interactions with other transforming events.

PTEN

PTEN was originally identified as a tumor suppressor that is frequently mutated or deleted in many cancers, including prostate (Salmena et al. 2008). The relevance of *PTEN* loss for prostate cancer was initially inferred from its location on chromosomal region 10q23, which frequently undergoes allelic loss in prostate cancer, as well as by its reduction or loss of expression in prostate tumors (Wang et al. 1998; Whang et al. 1998; McMenamin et al. 1999; Dong et al. 2007). Earlier studies had generated conflicting data regarding whether both alleles of *PTEN* are deleted in prostate cancer, or, if one allele is deleted, whether the remaining allele is mutated, or if the expression of PTEN protein is reduced, inactivated, or altered in subcellular localization. To resolve these issues, recent studies have investigated *PTEN* copy number, mutational status, and/or protein expression in primary or castration-resistant tumors using multiple experimental approaches (Verhagen et al. 2006; Schmitz et al. 2007; Sircar et al. 2009; Taylor et al. 2010). In combination with the consensus of previous reports, these studies support the conclusion that *PTEN* undergoes copy number loss as an early event in prostate carcinogenesis, and is correlated with progression to aggressive, castration-resistant disease. Interestingly, these studies have also suggested that low levels of *PTEN* activity may be retained in prostate cancer—an observation that parallels the haploinsufficiency of *NKX3.1* and the *p27* cell cycle regulator (Gao et al. 2004a; Abate-Shen et al. 2008), and which may reflect the relative indolence of prostate tumors.

Analyses of *Pten* deletion in genetically engineered mouse models have uncovered its cooperativity with inactivation of other key genes that are deregulated in prostate tumorigenesis, and have also provided insights into new therapeutic options for the treatment of prostate cancer. Germline loss of *Pten* in heterozygous mutants or conditional deletion in the prostate epithelium results in PIN and/or adenocarcinoma (Di Cristofano et al. 1998a; Podsypanina et al. 1999; Trotman et al. 2003; Wang et al. 2003). Inactivation of *Pten* has been shown to cooperate with loss of function of the *Nkx3.1* homeobox gene, up-regulation of the *c-Myc* proto-oncogene, or the *TMPRSS-ERG* fusion (Kim et al. 2002c, 2009; Carver et al. 2009; King et al. 2009). Additionally, investigations of *Pten* loss—together with perturbations of cell cycle regulators such as *p27*, $p18^{ink4c}$, and $p14^{arf}$ (Di Cristofano et al. 2001; Bai et al. 2006; Z Chen et al. 2009), or components of key signaling pathways such as *Rheb*, *TSC2*, and *Rictor* (L Ma et al. 2005; Nardella et al. 2008; Guertin et al. 2009)—have further emphasized the significance of haploinsufficiency in prostate cancer. Interestingly, the requirement of the mTORC2 complex as well as the p110β isoform of PI3K for tumor formation following *Pten* loss suggests that these signaling components may provide additional and/or alternative targets for therapeutic intervention (Jia et al. 2008; Guertin et al. 2009). Moreover, the observation that complete inactivation of *Pten* in mouse prostate tumors leads to cellular senescence (Z Chen et al. 2005) has led to the idea that novel therapeutic approaches might promote senescence for selective targeting of prostate tumor cells through knockdown of *Pten* function (Alimonti et al. 2010) or targeting of *Skp2* (Lin et al. 2010). Notably, *PTEN* reduction or loss in prostate cancer predisposes to the emergence of castration-resistant prostate cancer (Mulholland et al. 2006; Shen and Abate-Shen 2007). In particular, perturbation of *PTEN* expression in human prostate cancer cell lines or targeted deletion of *Pten* in mouse prostate cancers is sufficient for the development of castration resistance (Lin et al. 2004; Bertram et al. 2006; Gao et al. 2006b; Wu et al. 2006). While this may reflect the ability of *PTEN* to interact directly with AR, the mechanistic details by which *PTEN* loss promotes castration resistance remain to be resolved.

Signaling pathways—Akt/mTOR and MAPK signaling

As noted above, considerable evidence indicates that *Pten* loss of function results in up-regulation of the Akt/mTOR signaling pathway in prostate cancer, primarily through activation of Akt1 (Thomas et al. 2004; ML Chen et al. 2006; Mulholland et al. 2006; Shen and Abate-Shen 2007). Up-regulation of this pathway in prostate cancer can also take place through activating mutations of Akt1 (Boormans et al. 2008), or through activation of the p110β isoform of PI3K (Hill et al. 2010; Lee et al. 2010). The functional consequences of Akt/mTOR pathway activation are particularly relevant for castration-resistant prostate cancer, as has been shown in genetically engineered mouse models, in gain-of-function studies with orthotopic grafting or tissue recombination models, as well as in human cell lines (Majumder et al. 2003; Uzgare and Isaacs 2004; Gao et al. 2006a; Xin et al. 2006). Activation of Akt occurs primarily at the cell membrane, and is consequently sensitive to levels of cholesterol in prostate cancer cells (Zhuang et al. 2005; Adam et al. 2007); however, Akt has additional functions in the nucleus that are dependent on the levels of PML (Trotman et al. 2006). The consequences of Akt activation are mediated in part by activation of NF-κB signaling via stimulation of IKK (Dan et al. 2008). Conversely, functional studies in mouse models and correlative studies in human prostate cancer have implicated deregulated NF-κB signaling in mediating androgen responsivity, metastasis, and disease outcome (Fradet et al. 2004; Ismail et al. 2004; Lessard et al. 2006; Luo et al. 2007; Zhang et al. 2009).

In addition to Akt/mTOR signaling, Erk (p42/44) MAPK signaling is also frequently activated in prostate cancer, particularly in advanced disease, and is often coordinately deregulated together with Akt signaling (Abreu-Martin et al. 1999; Gioeli et al. 1999; Paweletz et al. 2001; Malik et al. 2002; Thomas et al. 2004; Kinkade et al. 2008). Simultaneous activation of these signaling pathways promotes tumor progression and castration resistance in prostate cancer cell lines and mouse models (Uzgare and Isaacs 2004; Gao et al. 2006a), while combinatorial inhibition of these pathways inhibits castration-resistant prostate cancer in genetically engineered mice (Kinkade et al. 2008). In contrast with Akt/mTOR signaling, the upstream events that lead to activation of Erk MAPK signaling are less well defined, but are thought to be linked to aberrant growth factor signaling (Gioeli 2005). Although mutations of *RAS* or *RAF* are rarely found in human prostate cancer, the pathway is frequently perturbed in advanced prostate cancers (Taylor et al. 2010). Notably, expression of activated forms of either *Raf* or *Ras* in the mouse prostrate epithelium results in MAPK activation and promotes cancer formation (Jeong et al. 2008; Pearson et al. 2009). Interestingly, a small percentage of aggressive prostate tumors contains a translocation of *B-RAF* or *C-RAF* that results in activation (Palanisamy et al. 2010), suggesting that perturbations of *Ras* or *Raf* signaling may occur in prostate cancer through mechanisms other than activating mutations.

Oncogenic tyrosine kinases

The deregulated expression of oncogenic tyrosine kinases has been studied extensively in many cancers, since these can represent targets for therapeutic intervention (Gschwind et al. 2004). In prostate cancer, aberrant tyrosine kinase signaling, particularly through Her2/Neu or SRC tyrosine kinases, has been implicated in aggressive disease, progression to metastasis, and castration resistance, and, consequently, has been implicated as a key therapeutic target in patients with advanced disease (Mellinghoff et al. 2004; Fizazi 2007). In particular, stimulation of AR signaling leads to activation of *SRC* in prostate cancer cells, which can lead to phosphorylation of AR, castration resistance, and cellular proliferation and invasiveness (Migliaccio et al. 2000; Agoulnik et al. 2005; Kraus et al. 2006). However, most functional analyses of *SRC* and other oncogenic tyrosine kinases have been limited to studies of prostate cancer cell lines in culture or in xenografts, and further insights will require analyses of in vivo models and correlative studies of clinical specimens.

Developmental signaling pathways

Molecular analyses of prostate development are likely to be informative for prostate carcinogenesis, as recent studies have shown that prostate tumors express a wide range of genes normally expressed during embryonic/neonatal organogenesis, suggesting that cancer progression reactivates embryonic developmental programs of gene expression (Schaeffer et al. 2008; Pritchard et al. 2009). In particular, elevated canonical Wnt signaling may play a role in the emergence of castration resistance (G Wang et al. 2008), while prostate cancer in mice can result from inactivation of *Apc* or overexpression of a constitutively active β-catenin together with activated *K-ras* (Bruxvoort et al. 2007; Pearson et al. 2009; Yu et al. 2009). In contrast, however, evidence from human tumors suggests that nuclear localization of β-catenin is inversely correlated with tumor progression (Horvath et al. 2005; Whitaker et al. 2008), suggesting that canonical Wnt signaling may not play a significant role in prostate cancer progression. With respect to the Hedgehog pathway, although there is considerable evidence that activation of Hedgehog signaling plays a significant role in prostate cancer progression, it remains unclear as to whether this occurs through an autocrine mechanism in epithelial cells (Karhadkar et al. 2004; Sanchez et al. 2004), or, alternatively, through paracrine signaling involving stromal components (Yauch et al. 2008; Shaw et al. 2009). Finally, paracrine FGF signaling has also been implicated in prostate cancer in mouse models, through either epithelial activation of FGFR1 or stromal overexpression of FGF10 (Acevedo et al. 2007; Memarzadeh et al. 2007). This up-regulation of FGF signaling may provide a mechanism for the activation of Erk MAPK pathway activity observed in prostate cancer progression.

Ezh2

The Polycomb group gene *EZH2* encodes a histone lysine methyltransferase that is frequently up-regulated in advanced prostate cancer, in some cases through gene amplification (Varambally et al. 2002; Saramaki et al. 2006), and is associated with aggressive tumors (Bachmann et al. 2006). *EZH2* expression is negatively regulated by miR-101, and miR-101 expression decreases during cancer progression, concomitant with somatic loss of one or both *miR-101* alleles (Zhao et al. 2007). Among the targets of *EZH2* is *NKX3.1*, which is repressed via expression of ERG and is dependent on H3K27 trimethylation (Kunderfranco et al. 2010). Other *EZH2* target genes in prostate cancer are associated specifically with metastasis, including *E-cadherin* (Cao et al. 2008) and *DAB2IP* (H Chen et al. 2005), which promotes prostate cancer metastasis through activation of Ras and NF-κB pathways (Min et al. 2010). However, *Ezh2* has also been shown to function in the cytoplasm to control actin polymerization in prostate and nonprostate cells (Su et al. 2005; Bryant et al. 2008), and therefore analyses of target genes may not fully explain *EZH2* function in cancer progression.

miRNAs

miRNAs regulate normal processes of growth and development as well as pathogenic processes associated with cancer, and analyses of their expression patterns have been effective for stratifying human cancers (Lu et al. 2005; Volinia et al. 2006). Expression profiling studies of human prostate tumors and xenografts have suggested that the expression patterns of miRNAs may distinguish indolent from aggressive tumors (Porkka et al.

2007; Ambs et al. 2008; Ozen et al. 2008; Coppola et al. 2009; DeVere White et al. 2009), and have implicated specific miRNAs in castration-resistant prostate cancer (Shi et al. 2007; Sun et al. 2009). Consistent with these findings, key enzymatic components of miRNA synthesis and processing such as Dicer are up-regulated during prostate tumor progression (Chiosea et al. 2006; Ambs et al. 2008; Poliseno et al. 2010a), while functional analyses of mice with conditional deletion of Dicer support a role for miRNAs in prostate epithelial proliferation (Zhang et al. 2010). Furthermore, miRNAs have specific roles in regulation of critical target genes, as the cluster miR-106b-25 negatively regulates *PTEN* expression (Poliseno et al. 2010a), while genomic loss of miR-101 leads to up-regulation of *EZH2* in prostate cancer progression (Varambally et al. 2008). In addition, functional analyses of the miR-15a–miR-16-1 cluster in regulating expression of *CCND1*, *WNT3A*, and *BCL2* in prostate cancer have provided an example of potential therapeutic benefit in restoring expression of miRNAs (Bonci et al. 2008), while detection of miRNAs in human plasma has been proposed as a platform for blood-based detection of human cancer (Mitchell et al. 2008). Notably, a recent study has shown that the expressed pseudogene *PTENP1* can regulate the expression of *PTEN* in prostate cancer by competing for miRNA binding (Poliseno et al. 2010b), demonstrating a new mechanism for regulating gene expression in human tumors.

AR function and castration resistance

AR and the emergence of castration resistance

AR is a nuclear hormone receptor whose signaling plays a key role in both normal prostate development and prostate cancer. The most abundant androgen is testosterone, which is synthesized by the testis and converted into the more active metabolite dihydrotestosterone in prostate tissue through the activity of 5a-reductase. In addition, the adrenal gland synthesizes minor androgen species, including androstenedione and dehydroepiandrosterone (DHEA), which can be converted into testosterone. In human patients, androgen deprivation therapy is usually performed by administration of gonadotropin-releasing hormone analogs and/or surgical castration (orchiectomy), often in combination with anti-androgens such as flutamide or bicalutamide.

Following androgen deprivation, the androgen dependence of prostate tissue is manifested by rapid cellular apoptosis and involution to the regressed state (Fig. 3). In culture, however, androgen-dependent prostate cell lines cease proliferation when androgens are removed, but do not undergo apoptosis (Watson et al. 2005; Gao et al. 2006a). In contrast, in tissue reconstitution experiments, the same prostate cell lines will display apoptosis following androgen deprivation, indicating that the apoptotic response is induced by stromal tissue (Gao et al. 2006a). These findings are consistent with earlier tissue reconstitution experiments that analyzed recombination of AR-null mutant epithelium with wild-type stroma (Kurita et al. 2001). Thus, androgen dependence of prostate epithelium in vivo requires paracrine activity of stromal AR, similar to the requirement for mesenchymal AR in epithelial–mesenchymal interactions during early prostate organogenesis (Shen and Abate-Shen 2007). Consistent with this conclusion, conditional deletion of AR in both epithelium and stroma of TRAMP mice resulted in smaller tumors with decreased proliferation relative to those formed after epithelial-specific AR deletion (Niu et al. 2008b).

In normal prostate epithelium, AR suppresses cellular proliferation, as probasin-Cre-mediated conditional deletion of AR leads to increased proliferation accompanied

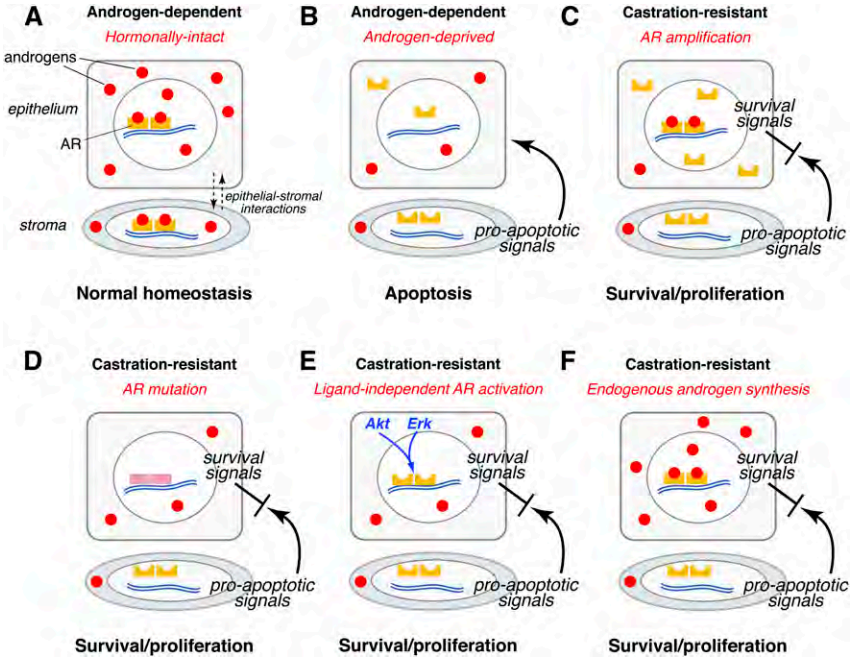

Figure 3. Role of AR in castration-resistant prostate cancer. (*A*) AR maintains homeostasis of both epithelial and stromal tissues in the normal prostate. (*B*) Following androgen ablation, stromal cells produce paracrine proapoptotic signals that act on neighboring epithelial cells, promoting regression of normal prostate. (*C–F*) Castration resistance can occur through a variety of molecular mechanisms, including AR amplification (*C*); gain-of-function mutation of AR mutation (*D*); ligand-independent AR activation by up-regulation of other signaling pathways, such as the Akt/mTOR and Erk MAPK pathways (*E*); or endogenous biosynthesis of androgens by tumor cells (*F*). Adapted from Shen and Abate-Shen (2007); © 2007 American Association for Cancer Research.

by decreased expression of differentiation markers (Wu et al. 2007). In prostate cancer, however, AR suppresses proliferation of basal cells, supports survival of luminal cells, and promotes metastasis, as shown by analyses of AR conditional deletion in the context of the TRAMP model (Niu et al. 2008a). This complex loss-of-function phenotype contrasts with more straightforward gain-of-function studies, as transgenic mice overexpressing wild-type AR under the control of the probasin promoter develop PIN (Stanbrough et al. 2001), while overexpression of an AR missense mutation results in prostate cancer (Han et al. 2005). Overall, it appears that AR is likely to play different cell type-specific roles in both normal and cancer cells, which are modulated by interactions with other key regulators of prostate epithelial fate. For example, Nkx3.1 negatively regulates AR transcription and signaling activity (Lei et al. 2006), while genomic analyses of AR enhancer-binding sites reveal likely interactions with Nkx3.1 and FoxA1, another key transcriptional regulator of prostate epithelial differentiation (Gao et al. 2005; He et al. 2010).

Retention of AR signaling in castration resistance

Even when prostate cancer progresses to castration resistance, AR activation and signaling remains sustained through a variety of mechanisms (Fig. 3; Taplin and Balk 2004; Attard et al. 2009a; Bonkhoff and Berges 2010). Notably, castration-resistant tumors express AR as well as AR target genes such as PSA, indicating that pathway activity is intact (Gregory et al. 1998). These findings have been most strongly supported by key experiments showing that xenografts that have been selected for castration resistance primarily differ from their parental androgen-dependent lines with respect to levels of AR expression (Chen et al. 2004). Thus, androgen signaling switches from a paracrine mechanism involving the stroma in androgen-dependent cells to an autocrine mechanism in castration resistance (Gao et al. 2001).

Several molecular mechanisms have been described for the ability of AR to retain signaling activity in castration-resistant prostate cancer. These mechanisms include the amplification of AR gene copy number in approximately one-third of castration-resistant carcinomas (Visakorpi et al. 1995; Koivisto et al. 1997; Linja et al. 2001). Another 10%–30% of tumors have gain-of-function mutations of AR that may confer increased protein stability, greater sensitivity to androgens, novel responses to other steroid hormones, ligand-independent activity, or increased recruitment of AR coactivator proteins (Taplin et al. 1995, 2003; Zhao et al. 2000; Robzyk et al. 2007; Brooke et al. 2008; Steinkamp et al. 2009). In addition, recent studies have shown that expression of alternative splice isoforms encoding constitutively active AR variants also occurs in castration-resistant cancer (Dehm et al. 2008; Guo et al. 2009; Hu et al. 2009). Finally, an unusual mechanism for increased AR signaling activity is the endogenous expression of androgen synthetic enzymes by tumor tissue, which can lead to de novo androgen synthesis or conversion of weaker adrenal androgens into testosterone and dihydrotestosterone (Titus et al. 2005; Stanbrough et al. 2006; Locke et al. 2008; Montgomery et al. 2008).

Ligand-independent activation of AR activity can also take place through activation of growth factor signaling pathways. Notably, up-regulation of the PI3K pathway through *Pten* deletion appears to be particularly effective, as PIN lesions in *Nkx3.1; Pten* double-mutant mice display castration resistance prior to carcinoma formation (Gao et al. 2006b). Furthermore, analysis of androgen-dependent cell lines in tissue reconstitution assays has shown that castration resistance can be induced by activation of the PI3K pathway, and is synergistically enhanced by up-regulation of MAPK signaling, but remains dependent on AR function (Gao et al. 2006a; Jiao et al. 2007). At the molecular level, growth factor signaling can up-regulate AR transcriptional activity through increased tyrosine phosphorylation, or perhaps elevated ubiquitination of AR (Guo et al. 2006; Xu et al. 2009).

Finally, castration resistance can be enhanced through an increased inflammatory response. For example, production of interleukin-1β by infiltrating macrophages can lead to derepression of the AR corepressor complex in prostate tumor cells, thereby converting AR antagonists into agonists (Zhu et al. 2006). In addition, production of inflammatory cytokines by B lymphocytes can lead to nuclear translocation of IKKα and castration resistance in mouse prostate tumor cells and allografts (Luo et al. 2007). Moreover, analyses of TRAMP mice and cell lines have shown that nuclear IKKα can enhance prostate cancer metastasis through down-regulation of Maspin (Luo et al. 2007). Consequently, the emergence of castration resistance and metastasis may be coordinately linked at the molecular level through interactions with the tumor microenvironment.

Overall, these findings suggest that AR target genes and regulatory networks should be similar in androgen-dependent and castration-resistant prostate cancer. This conclusion has been supported by expression profiling of tumors with and without neoadjuvant androgen ablation prior to radical prostatectomy, which showed that castration-resistant tumors displayed up-regulation of AR, androgen synthetic enzymes, and known AR target genes (Holzbeierlein et al. 2004). However, recent genomic chromatin immunoprecipitation studies have shown that AR activity in castration-resistant prostate cancer is not identical to that displayed by AR in androgen-dependent cells. In particular, there is a significant alteration of genomic AR-binding targets and associated epigenetic chromatin marks in castration-resistant prostate cancer cell lines, resulting in up-regulation of M-phase-associated cell cycle genes (Q Wang et al. 2009). These findings suggest that AR-interacting proteins and/or histone-modifying enzymes may play a significant role in mediating castration resistance.

At present, it is unclear when castration resistance normally arises within prostate tumors. The conventional "adaptation" model proposes that castration-resistant cells arise through genetic/epigenetic conversion of previously androgen-dependent cells during conditions of androgen deprivation, while the alternative "clonal selection" model suggests that emergence of castration resistance reflects the proliferation of a previously quiescent

population of rare castration-resistant cells within an otherwise androgen-dependent tumor (Isaacs and Coffey 1981). Although the former model represents the prevailing view, evidence for the latter model has been provided in studies of the onset of castration resistance in TRAMP mice (Gingrich et al. 1997), as well as through limiting dilution and fluctuation analyses of an androgen-dependent xenograft (Craft et al. 1999a). In addition, analysis of localized human prostate tumors suggests that rare AR mutations can be detected prior to androgen deprivation therapy (Gaddipati et al. 1994; Tilley et al. 1996; Bergerat and Ceraline 2009). Furthermore, the finding that castration-resistant cells such as CARNs (castration-resistant Nkx3.1-expressing cells) represent a cell of origin for prostate cancer also favors a clonal selection model (X Wang et al. 2009) in which the rare castration-resistant population might also correspond to putative cancer stem cells. Thus, while some mechanisms of castration resistance may represent an adaptive response to androgen deprivation therapy, in many cases, increased AR activity may be selected prior to treatment during prostate cancer progression.

Prostate stem cells and tumor-initiating cells (TICs)

Localization of adult stem cells

A tissue stem cell can be defined as a progenitor that is multipotent, being capable of giving rise to distinct cell types of the tissue of interest, and also able to self-renew by maintaining the stem cell phenotype in progeny following cell division (Rossi et al. 2008). In the case of the adult prostate, the existence of epithelial stem cells is implied by the ability of the adult prostate to undergo repeated cycles of extensive regression in response to androgen deprivation, followed by full regeneration following androgen restoration. Consequently, the prostate epithelium should contain a long-term resident pool of stem cells that are castration-resistant (Isaacs 1985). Notably, the majority of luminal cells are androgen-dependent and undergo apoptosis following castration, while most basal and neuroendocrine cells survive and are castration-resistant (English et al. 1987; Evans and Chandler 1987).

Most studies of prostate epithelial stem cells have relied on flow cytometry to purify subsets of epithelial cells based on cell surface marker expression, and have explored their progenitor potential in cell culture or transplantation assays (Lawson and Witte 2007; Kasper 2008). In particular, subpopulations of prostate basal cells isolated using cell surface markers can display bipotentiality and self-renewal in cell culture as well as tissue reconstitution assays (Richardson et al. 2004; Burger et al. 2005; Xin et al. 2005; Lawson et al. 2007; Goldstein et al. 2008). For example, isolation of a $Lin^{-}Sca\text{-}1^{+}CD49f^{+}$ population results in a 60-fold enrichment for stem cells (Lawson et al. 2007). Further enrichment can be obtained using the Trop2 marker, which also allows isolation of a stem cell-enriched $Lin^{-}CD49f^{+}Trop2^{+}$ fraction from human prostate epithelium (Goldstein et al. 2008). In independent studies, $\alpha_2\beta_1$ $integrin^{hi}CD133^{+}$ basal cells also correspond to an enriched stem cell fraction in human prostate epithelium (Richardson et al. 2004). Finally, single $Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$ cells, which are predominantly basal in mice and are exclusively basal in humans, have been reported to reconstitute prostatic ducts in renal grafts (Leong et al. 2008).

In contrast, other data have supported a luminal localization of prostate epithelial stem cells, primarily with respect to prostate regeneration. In particular, analyses of grafted tissue from *p63*-null mice have demonstrated the formation and serial regression/regeneration of prostate tissue in the absence of basal cells (Kurita et al. 2004). Furthermore, recent studies have identified a rare luminal population of CARNs in the regressed prostate epithelium that displays stem cell properties during prostate regeneration (X Wang et al. 2009). In particular, in vivo genetic lineage marking showed that CARNs display bipotentiality and long-term self-renewal during prostate regeneration, and are also capable of reconstituting prostatic ducts following single-cell transplantation.

At present, it is difficult to ascertain the potential overlap as well as lineage relationships of the various candidate stem cells that have been identified, in part due to the distinct methodologies and assays employed (Fig. 4). In addition, individual cell surface markers may lack specificity for stem/progenitor cells, as has been

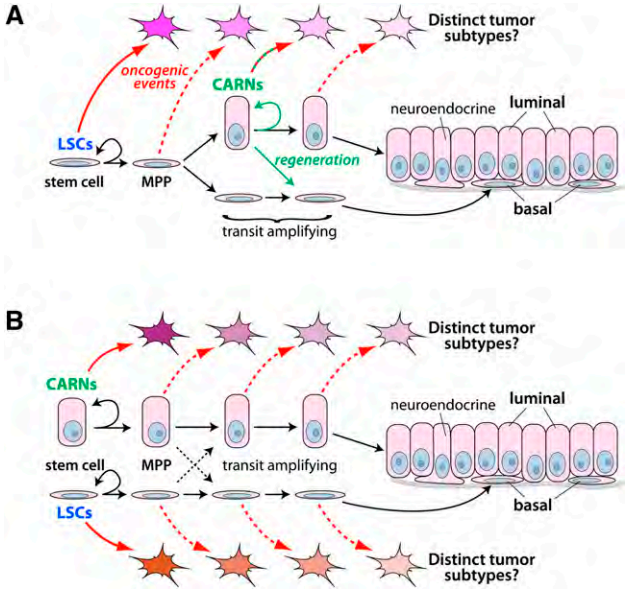

Figure 4. Lineage hierarchy in the prostate epithelium and the cell of origin for prostate cancer. Two possible lineage relationships for the adult prostate epithelium are shown, together with the potential roles of $Lin^{-}Sca\text{-}1^{+}CD49f^{+}$ cells (LSCs) and CARNs. Different cell types of origin in the lineage hierarchy might then generate distinct tumor subtypes following oncogenic transformation (red arrows). (*A*) In this model, LSCs correspond to stem cells, and CARNs correspond to a luminal progenitor that acquires stem cell properties in the context of prostate regeneration (green arrows), thus corresponding to a facultative stem cell. (*B*) An alternative model is that LSCs and CARNs correspond to independent stem cells that maintain basal and luminal populations, respectively. Adapted from X Wang et al. (2009); © 2009 Nature.

suggested for CD133 (Shmelkov et al. 2008). It is also notable that existing cell culture assays are performed under conditions that select strongly against the growth of luminal cells (Peehl 2005), resulting in a significant bias toward outgrowth of basal cells and basal cell differentiation in assays such as prostasphere formation. Thus, in the absence of comprehensive in vivo approaches to investigate stem cell properties, the present data suggest that there may be multiple independent stem cell populations within the adult prostate epithelium.

Cell of origin

The tissue localization of prostate epithelial stem cells is highly relevant for investigating the putative cell type(s) of origin for prostate cancer (Lawson and Witte 2007; Kasper 2008; Maitland and Collins 2008). A cell of origin can be defined as a normal tissue cell that can be oncogenically transformed to give rise to a cancer; thus, the cell of origin refers to a cell or cell type that is found in normal untransformed tissue. In principle, cancer could result from transformation of a rare stem cell, and/or could result from transformation of a more restricted cell type (such as a transit/amplifying cell) and its "dedifferentiation" to acquire self-renewal properties characteristic of stem cells (Fig. 4). Indeed, differences in the cell of origin in the stem cell lineage hierarchy have been proposed to represent the basis for distinct tumor subtypes for breast cancer (Visvader 2009).

Given the luminal phenotype of human prostate cancer, the cell of origin should correspond to either a luminal cell, or a basal progenitor that can rapidly differentiate into luminal progeny following oncogenic transformation. A basal cell of origin has been suggested by analyses of *Pb-Cre4; Pten*$^{flox/flox}$ mice, which display an expansion of basal cells as well as intermediate cells coexpressing basal and luminal markers in tumors (S Wang et al. 2006). More recently, a comparison of basal and luminal epithelial populations isolated by flow cytometry from the mouse prostate has shown that basal populations are readily transformed by lentiviral expression of ERG and AR in tissue reconstitution experiments, whereas luminal cells are not transformed (Lawson et al. 2010). Importantly, analogous reconstitution assays using normal epithelial cells isolated from the human prostate have shown that transformed basal cells can generate prostate adenocarcinomas with luminal phenotypes (Goldstein et al. 2010).

In contrast, studies of *PSA-Cre; Pten*$^{flox/flox}$ mice have suggested a rare luminal Clu^{+}Tacstd2^{+}Sca-1^{+} population as corresponding to the cell of origin in this model (Korsten et al. 2009). Consistent with these findings, detailed phenotypic analysis of *Probasin-Myc* and *Nkx3.1-Myc* transgenic mouse lines also suggests that PIN and prostate cancer originates from luminal cells (Iwata et al. 2010). Notably, CARNs correspond to luminal cells of origin for prostate cancer in mouse models, as evidenced by targeted deletion of Pten resulting in high-grade PIN and invasive carcinoma following androgen repletion and prostate regeneration (X Wang et al. 2009). Additional evidence is suggested by detailed histopathological analysis of MYC expression in high-grade PIN samples, which still retain basal cells, which shows that MYC up-regulation is associated exclusively with luminal cells, and is not detected in their basal neighbors (Gurel et al. 2008); similar findings have also been reported with respect to telomere shortening (Meeker et al. 2002). Also in favor of a luminal cell of origin is the recent finding that AR mediates formation of the *TMPRSS2-ERG* fusion in human prostate cancer cells (Lin et al. 2009; Mani et al. 2009; Haffner et al. 2010), suggesting that initiating events take place in AR-expressing luminal cells. Thus, based on the available evidence, prostate cancer can indeed arise from distinct cell types of origin, but it remains unclear whether different cells of origin are used in human prostate cancer initiation, or whether they might result in differing molecular subtypes.

Identification of TICs

The cancer stem cell model proposes that cell populations within a tumor have a hierarchical organization, in which a stem cell-like population gives rise to more differentiated derivatives that lack tumor-initiating and/or long-term self-renewal capability (Reya et al. 2001; Pardal et al. 2003; Wicha et al. 2006; Visvader and Lindeman 2008; Marotta and Polyak 2009; Rosen and Jordan 2009). This model has strong translational and clinical relevance, since it would likely have several implications for prostate cancer treatment. First, the identification of appropriate markers would allow the correlation of prostate cancer stem cell status in tumors with histopathology and clinical outcomes, and might also serve as accurate surrogates for the efficacy of cancer treatments. Second, targeted therapeutics for cancer stem cells might be superior to conventional therapies, which usually target cellular proliferation in the bulk tumor, while cancer stem cells may be relatively resistant due to a lower proliferative rate. Finally, the assessment of cancer stem cell numbers and molecular properties among circulating tumor cells might have prognostic value for the risk of metastatic disease, since the ability of circulating tumor cells to generate secondary metastases presumably requires self-renewing cancer stem cells.

The cancer stem cell model is consistent with the observed phenotypic heterogeneity found in many tumors, including prostate adenocarcinoma. In contrast, a stochastic or clonal evolution model of tumor development suggests that the phenotypic heterogeneity of tumors is due to variations in the genetic or epigenetic composition of tumor subpopulations, but that these subpopulations are not hierarchically organized and have similar tumor-initiating ability under appropriate circumstances (Adams and Strasser 2008; Shackleton et al. 2009). In many experimental contexts, cancer stem cells are identified in assays for TICs, using xenotransplantation to isolate cancer cells that can form a tumor after grafting, most rigorously after transplantation of a single cell. However, recent work has questioned the interpretation of such studies, since technical improvements in xenotransplantation can yield significant increases in efficiency, with up to 25% of melanoma cells displaying

tumor-initiating properties (Quintana et al. 2008). These and other studies continue to engender doubt as to the existence of cancer stem cells in many solid tumors (Hill 2006; Shackleton et al. 2009).

Flow cytometry approaches to purifying subsets of epithelial cells based on cell surface marker expression have been combined with xenograft assays to identify putative TICs isolated from mouse prostate cancer models as well as human prostate cancer specimens (Lawson and Witte 2007; Kasper 2008). In the case of mouse prostate cancer, $Lin^{-}Sca\text{-}1^{+}CD49f^{+}$ cells from *PbCre4*; *Pten*$^{flox/flox}$ mice have been shown to have tumor-initiating properties in renal graft and sphere-forming assays, suggesting marker conservation between normal stem cells and cancer stem cells (Mulholland et al. 2009). In human prostate cancer, CD44 has been used as a marker to enrich for TICs from established xenografts (Patrawala et al. 2006), while further enrichment of TICs was obtained in a subsequent study by sorting for $\alpha_2\beta_1$ integrin$^{hi}CD44^{+}$ cells (Patrawala et al. 2007). Finally, enrichment of $CD133^{+}\alpha_2\beta_1$ integrin$^{hi}CD44^{+}$ cells from primary prostate tumor biopsies resulted in identification of cells with increased invasiveness and clonogenicity in culture (Collins et al. 2005), while molecular analyses of $CD133^{+}\alpha_2\beta_1$ integrin^{hi} cells revealed a potential cancer stem cell signature that is enriched for components of the JAK–STAT, Wnt, and focal adhesion pathways (Birnie et al. 2008). To date, however, the successful use of cell surface markers to isolate cell populations from primary human prostate cancers with tumor-initiating capabilities in grafting assays has not yet been reported.

Despite these promising findings, it remains unclear whether normal stem cells and cancer stem cells should display conserved marker expression, or whether the markers used display specificity for putative cancer stem cells. Second, the candidate TICs isolated to date display prevalent basal cell differentiation in vivo and in vitro, which is unexpected, since the primary tumors from which these cells were derived presumably lack basal cells. Finally, the putative TICs lack expression of AR, which is surprising given the strong selection for AR activity throughout prostate cancer progression, and the known mechanisms for castration resistance (Sharifi et al. 2006). These concerns suggest that authentic prostate cancer stem cells have not yet been definitively identified.

Translational applications

In recent years, principal areas of translational research on prostate cancer have focused on (1) understanding the dietary/lifestyle/environmental factors that influence prostate carcinogenesis, and identifying strategies to delay its onset or progression; (2) identifying biomarkers that distinguish indolent versus aggressive forms of the disease, and the application of such biomarkers for patient stratification; and (3) developing new therapeutic approaches for the treatment of castration-resistant prostate cancer, as well as for prevention of bone metastases. For instance, one example of a novel therapeutic approach that may be promising is the use of immunotherapy, as exemplified by the recent FDA approval of a therapeutic vaccine (Provenge) for advanced prostate cancer patients (Harzstark and Small 2007; Morse and Whelan 2010). Below, we briefly highlight major directions for translational research, focusing on how they can benefit from basic research, recent technological advances, and/or the application of robust preclinical models for in vivo analyses.

Dietary and lifestyle factors in cancer prevention

Epidemiologic investigations support the idea that dietary/lifestyle factors are major contributors of population differences in the occurrence of clinical prostate cancer (Kolonel et al. 2000, Kolonel 2001). In particular, dietary/lifestyle differences may account for the considerable difference in incidence of clinical prostate cancer between Asian and American populations, reflecting a shift in the rate of cancer detection by ~10 years; notably, this discrepancy in cancer rate disappears when Asians immigrate to Western countries (Hanenszel and Kurihari 1968; Dunn 1975). However, the molecular/mechanistic bases for these differences have not been fully explained.

Considerable data support the hypothesis that dietary/lifestyle factors affect prostate cancer incidence by promoting chronic inflammation and/or oxidative stress, ultimately leading to DNA damage, epigenetic modifications, or other perturbations associated with cancer initiation (De Marzo et al. 2007a; Nelson 2007). This model has consequently emphasized the role of antioxidants and anti-inflamatory agents in protection against prostate cancer (DeWeese et al. 2001). Some prevention trials testing this model have been successful, including one showing that consumption of large quantities of tomato, which contain the potent antioxidant lycopene, results in reduced prostate cancer incidence (Chen et al. 2001). However, other trials have not shown that supplementation with antioxidants will reduce prostate cancer risk (Kirsh et al. 2006). A particular disappointment was the outcome of the SELECT trial, which found no benefit to supplementation with selenium and vitamin E (Lippman et al. 2009).

Additional studies have addressed the potential efficacy of antioxidants, anti-inflammatory agents, and/or other dietary factors by using epidemiological findings to investigate preclinical mouse models. For example, based on an extensive body of literature indicating that dietary restriction is anti-tumorigenic, analyses of dietary restriction or low-fat diets on cancer progression in genetically engineered mice has revealed the PI3K–Akt signaling pathway as a molecular target for these dietary interventions (Berquin et al. 2007; Kobayashi et al. 2008; Kalaany and Sabatini 2009). Another promising agent is vitamin D, which has been suggested by ample epidemiological evidence to protect against tumorigenesis, but has displayed variable efficacy in clinical trials (Deeb et al. 2007). Notably, analyses in genetically engineered mice that have shown that the timing of vitamin D administration is critical, as its beneficial effects are only realized early in cancer progression, as it promotes

expression of the vitamin D receptor in prostate epithelial cells (Banach-Petrosky et al. 2006). These examples highlight the importance of integrating epidemiological analyses with systematic evaluation of mechanisms in preclinical models for effective design and implementation of dietary interventions for cancer prevention.

Biomarker discovery

PSA testing has revolutionized the diagnosis of prostate cancer, since it is now possible to detect most prostate tumors at early stages, unlike other cancers that lack a straightforward method for early detection. However, the early detection of prostate cancer needs to be augmented by improved biomarkers that can stratify patients in conjunction with Gleason grading. The search for effective biomarkers has included gene expression profiling, miRNA expression profiling, serum proteomics, and metabolomics. The latter represents a promising new approach that may allow for the development of noninvasive urine tests for cancer metabolites to detect prostate and other cancers (Sreekumar et al. 2009). More generally, the investigation of potential urine biomarkers has led to the identification of PCA3 (prostate cancer antigen 3), a promising marker for predicting disease outcome (Ploussard and de la Taille 2010).

However, to date, few if any biomarkers are now being used that can predict disease outcome more effectively than Gleason score alone. In principle, suitable combinations of markers may be successful in cumulatively predicting outcome, as enabled by new technologies such as molecular systems pathology (Cordon-Cardo et al. 2007). Alternatively, system biology approaches that identify master regulatory genes of disease progression may enable the effective stratification of patients, as has been applied for other cancer types (Carro et al. 2009). Finally, comprehensive oncogenomic approaches that integrate gene expression and copy number analyses may identify new biomarkers for predicting disease outcome (Taylor et al. 2010).

Manipulating AR signaling for prevention and treatment

The essential role of AR signaling for the development of prostate cancer provided the rationale for a large-scale prevention trial that evaluated the 5α-reductase inhibitor finasteride for prevention of prostate cancer (Higgins and Thompson 2004). The results of this trial were encouraging, since they showed a 24% reduction in prostate cancer incidence, which has led to the recommendation of finasteride administration for men in high-risk categories. As a cautionary note, however, a subset of patients in this trial appeared to develop more aggressive disease (Lucia et al. 2007), which may reflect a selection for men predisposed to limiting levels of androgens, as has been suggested by studies of limiting androgen levels for cancer progression in genetically engineered mice (Banach-Petrosky et al. 2007).

AR has also been a primary target for treatment of patients with advanced disease. Based on the central role of AR in castration resistance, novel AR pathway inhibitors could potentially provide important therapeutics for advanced prostate cancer (Attar et al. 2009; Y Chen et al. 2009; Knudsen and Scher 2009). In this regard, a second-generation AR antagonist, MDV3100, which completely lacks agonist activity and binds AR with greater affinity than bicalutamide, has provided new insights into castration resistance, and has given promising results in mouse models and in a human phase 1–2 trial (Tran et al. 2009; Scher et al. 2010). Other agents that target the N-terminal transcriptional regulatory region of AR are now being evaluated in cell lines and mouse models (Andersen et al. 2010). Another promising AR pathway antagonist is abiraterone acetate, which inhibits the activity of CYP17, an enzyme required for two steps in androgen biosynthesis, and has shown promising results in initial clinical trials (Attard et al. 2009b; Y Chen et al. 2009).

Targeting signaling pathways in treatment of advanced disease

For reasons that are poorly understood, the therapeutic benefits of standard chemotherapy regimens are limited in patients with advanced prostate cancer, although improvements have been made in the past several years (Calabro and Sternberg 2007; Petrylak 2007). Therefore, recent approaches have been aimed at targeting signaling pathways activated in advanced prostate cancer, including the Akt/mTOR and MAPK signaling pathways. The evaluation of Rapamycin and related compounds (Rapalogs) that target mTOR signaling in preclinical trials in genetically engineered mutant mice and in human clinical trials suggest that these may not be effective as single agents (Sawyers 2003; Garcia-Echeverria and Sellers 2008; Morgan et al. 2009). However, combination therapy using Akt/mTOR inhibitors in conjunction with first-line chemotherapy or agents that target other key signaling pathways such as the Erk MAPK pathway may be highly effective, as has been suggested by preclinical studies in which combination therapy effectively blocks castration-resistant prostate cancer in mice (Kinkade et al. 2008). Thus, the development of combination therapy for treatment of advanced prostate cancer will likely benefit from evaluation in robust preclinical models.

Perspectives and conclusions

Considering the tremendous progress made in the past 10 years, we envision continuing advances over the next decade in areas of research that will facilitate effective strategies for the prevention, diagnosis, and treatment of prostate cancer. Among the challenges for future studies will be to integrate epidemiological studies with molecular investigations and clinical analyses to gain fundamental insights into how environmental, dietary, and lifestyle influences contribute to the development of prostate cancer, and to identify the molecular factors that are altered by these influences and how they can be modified by appropriate dietary or chemical interventions. Of paramount importance will be the effective

diagnosis of men that have prostate cancer, and their stratification into high-risk and low-risk groups for treatment management. Thus, biomarker discovery will likely represent a considerable emphasis for future research, perhaps focused on identification of master regulator genes that can provide accurate readouts of signaling pathways associated with disease progression. Moreover, considering that prostate cancer is fairly indolent, the development of treatment approaches that delay its onset or progression is likely to have a significant impact on outcome. Finally, more effective strategies will be necessary for preventing the transition to lethal forms of prostate cancer, which will require a deeper understanding of the mechanisms underlying castration-resistant prostate cancer and the bone tropism of prostate cancer metastasis. Thus, while our knowledge of the molecular genetics of prostate cancer has greatly expanded in the past decade, much work remains to be done to enhance the overall rate of prostate cancer survival.

Acknowledgments

We apologize to our colleagues for our inability to discuss and cite numerous important studies, due to length constraints. We thank Robert Cardiff and Alexander Borowsky for assistance with Figure 2, and Arul Chinnaiyan, Angelo De Marzo, Edward Gelmann, and Charles Sawyers for helpful discussions. This work was supported by grants to M.M.S. and C.A.-S. from the NIH, DOD Prostate Cancer Research Program, and NCI Mouse Models of Human Cancer Consortium.

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